República Federativa do Brasil
Ministério de Ciência, Tecnologia e Inovações - MCTI
Instituto Nacional de Pesquisas da Amazônia - INPA
Programa de Pós-Graduação em Ciências de Florestas Tropicais - PPGCFT

MASTER'S DISSERTATION

Propagation, morphophysiological, and anatomical aspects of *Bertholletia excelsa* genotypes

ELMER VIANA GONÇALVES

Manaus, AM
2023

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DE FLORESTAS TROPICAIS

ATA DE DEFESA PÚBLICA DE DISSERTAÇÃO - MESTRADO

Ata da Defesa hibrida de **ELMER VIANA GONÇALVES**, ocorrida no dia 14/08/2023, na sala da de aula 01 do PPG CFT, INPA, Campus III e via plataforma de vidoconferência Google Meet.

Aos 14 de agosto de 2023, às 14h00 (horário de Manaus/AM), realizou-se a Defesa Pública de Dissertação de **ELMER VIANA GONÇALVES**, aluno do Programa de Pós-Graduação *Stricto sensu* em Ciências de Florestas Tropicais, intitulada "**Propagation, morphophysiological and anatomical aspects of *Bertholletia excelsa* genotypes**", sob a orientação do Dr. José Francisco de Carvalho Gonçalves e coorientação dos doutores, Dr. Adamir da Rocha Nina Junior, Dra. Karen Cristina Pires da Costa, Dra. Josiane Celerino de Carvalho, em conformidade com o Art. 52 do Regimento Geral da Pós-Graduação do Instituto Nacional de Pesquisas da Amazônia (MCTI/INPA) e Art. 67 do Regimento Interno do Programa de Pós-Graduação em Ciências de Florestas Tropicais, como parte das atividades para conclusão e obtenção do Título de Mestre em Ciências de Florestas Tropicais. A **Banca Examinadora** foi constituída pelos seguintes membros: Luciedi de Cassia L Tostes (IEPA), Flavia Camila Schimpl (IFAM), Patricia M. Albuquerque (UEA). Suplentes, e tendo como suplentes os seguintes membros: Eva Maria Alves Cavalcanti Atroch (UFAM), Roberval M. B. Lima (EMBRAPA), Ângela Maria da Silva Mendes (UFAM), Roberto Kyrmayr Jaquetti (INPA). O Presidente da Banca Examinadora deu início à sessão e informou os procedimentos do exame. O aluno fez uma exposição do seu estudo e ao término foi arguido oralmente pelos membros da Comissão. Após as arguições os membros da banca se reuniram para avaliação e chegaram ao seguinte parecer:

Nome	Parecer		Assinaturas
Luciedi de Cassia Leoncio Tostes	(X) Aprovou	( ) Reprovou	
Flavia Camila Schimpl	(X) Aprovou	( ) Reprovou	
Patrícia Melchionna Albuquerque	(X) Aprovou	( ) Reprovou	

Menção: ( ) "Com Distinção" ( ) "Com Louvor" ( ) "Com Distinção e Louvor"

Nada mais havendo a tratar, foi lavrada a presente Ata que, após lida e aprovada, foi assinada pela Coordenação

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Sinopse:

Identificação de marcadores morfofisiológico de sementes e plântulas, e morfoanatômicos de folhas para a diferenciação de genótipos selvagens e cultivados de *Bertholletia excelsa;* estabelecimento de protocolo de cultivo *in vitro* de embriões zigóticos de *B. excelsa*; caracterização morfológica e anatômica foliar de genótipos selvagem e cultivado de *B. excelsa*, durante germinação nos ambientes de cultivo *in vivo* e *in vitro*.

Palavras-chave: Fisiologia de sementes, marcadores morfoanatômicos, germinação, tecnologia de sementes

A Deus, à Espiritualidade e aos seres de luz que me guiaram em todo o meu percurso;

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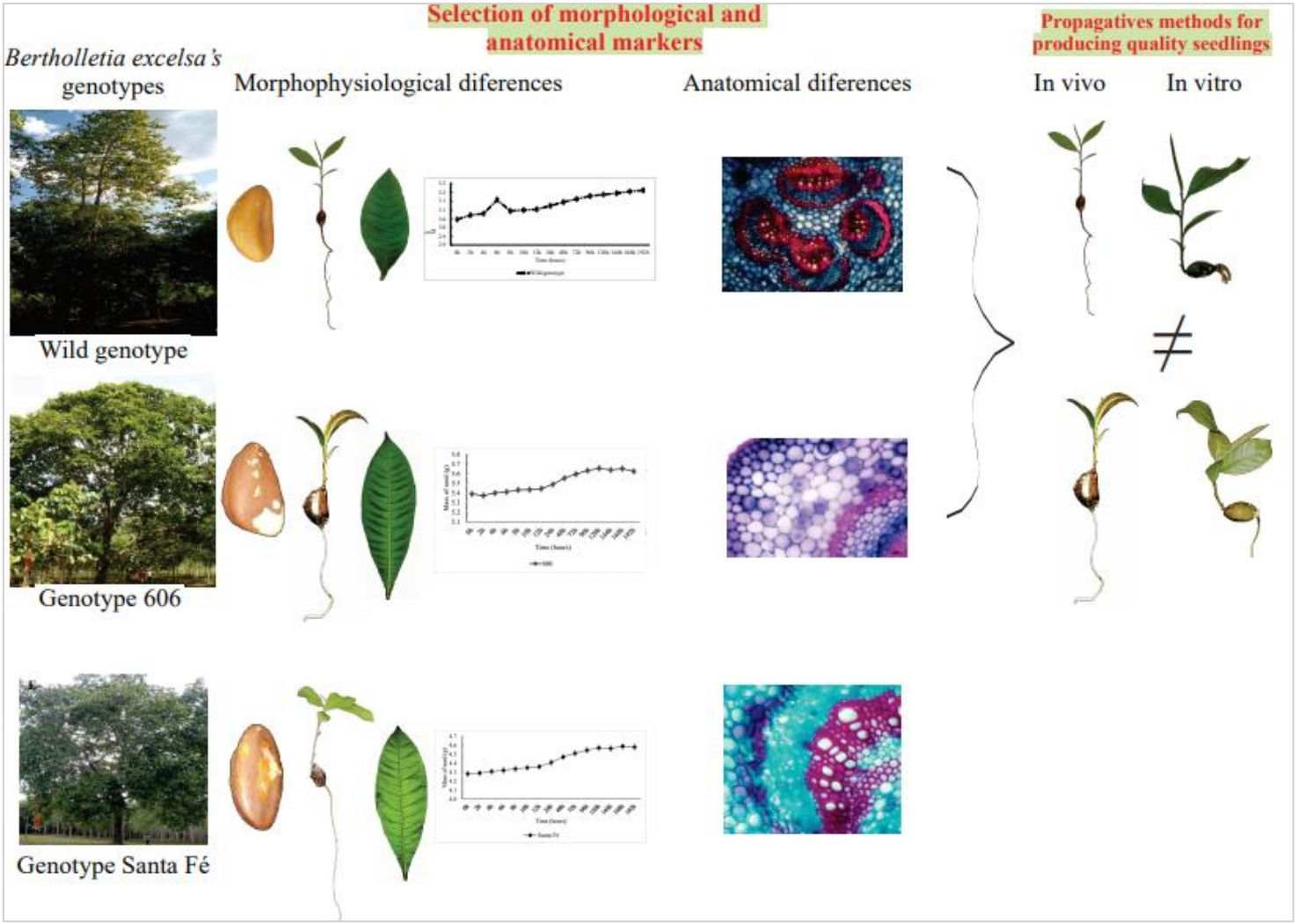
GRAPHICAL ABSTRACT



GENERAL ABSTRACT

In recent years, there has been an increasing urgency to recover degraded areas, especially in the Amazon region. This brings to light the interest in using native forest species for this purpose, focusing on the production of quality seeds and seedlings. One of the species that serves this purpose is the Brazil nut tree (*Bertholletia excelsa* Bonpl.), due to its high plasticity in developing and maintaining itself in different soil and climate conditions, and because of her being endangered, it needs technologies related to the propagation and conservation of genetic material to guarantee its existence. However, the main form of propagation of the species occurs via seeds, which has the limitation of low germination rate, and because it is slow and uneven in the natural environment and in the nursery, mainly due to the integumentary and physiological dormancy of the seed, and fungal contamination. An alternative to this is the in vitro micropropagation of zygotic embryos, which provides a high rate of germination, on a large scale in an aseptic environment. Germination is strongly influenced by genotype, since it is associated with the expression of morphophysiological characteristics of seeds and anatomical leaves. Among the genetic improvement initiatives of the species, some genotypes were generated, of which the 606 and Santa Fé stand out, the first of which stands out due to its characteristics of matrix growth, and the second for the productivity of seeds and fruits. Therefore, this work seeks to identify morphophysiological and anatomical markers that differentiate different genotypes of Brazil nut trees, as well as to investigate more efficient propagation methods for the production of seedlings of the species. For this, seeds of the two genotypes were collected at Amazonas state, and of a wild genotype in the state of Pará, which had their integuments removed, and then were taken to the Laboratory of Plant Physiology and Biochemistry, where their morphophysiological variables were measured, sown in expanded vermiculite, and the zygotic embryos of the 606 and wild genotypes were inoculated in full MS (Murashige Skoog) medium; after the complete seedling, the leaf morphoanatomy was described. As for the morphophysiology, all the genotypes had significant differences, and morphoanatomical differences were also detected in the leaves of the seedlings. Genotype 606 stood out in terms of seed size, germination rate, and parameters involving germination speed. With these variables, it was possible to group the two genotypes in a principal component analysis (PCA), where the variable most associated with Santa Fé was the relative amount of water absorbed, while for genotype 606 it was the percentage of germination. As for the morphoanatomical characteristics of the seedlings leaves, there was a difference in the morphology between the wild genotype, and the cultivated genotypes. These data show significant differences between the genotypes, facilitating the differentiation between them, and allowing choosing, within the conditions of this study, genotype 606 as the most suitable to produce quality seeds and seedlings. The *in vitro* micropropagation proved to be promising in relation to the decrease in germination time and seedling formation, which there was a decrease of 74 days for the formation of a seedling, for the wild genotype, and 161 days for the 606, when comparing in vivo and in vitro cultivation. Germination morphology between genotypes and propagation forms was different, since the zygotic embryos showed a more intense green color, while in the ex vitro culture, only some portions of the seeds turned green, and moreover, some explants of the wild type did not develop roots, only aerial part. Therefore, for Brazil nut trees, it is possible to use the markers related to seed biometry and germination, as well as the morphology of the leaf blade to identify the wild genotype, the cultivated genotype, and in addition, in vitro micropropagation proved to be a promising method for the production of Brazil nut seedlings in a shorter time than that used in propagation via seeds in vivo.

Keywords: Brazil nut tree, imbibition curve, seedling production, seed technology, tissue culture

RESUMO GERAL

Nos últimos anos, tem havido uma urgência crescente para recuperar áreas degradadas, especialmente na região amazônica. Isso traz à tona o interesse em utilizar espécies florestais nativas para esse fim, com foco na produção de sementes e mudas de qualidade. Uma das espécies que atende a esse propósito é a castanheira-do-brasil *(Bertholletia excelsa* Bonpl.), devido à sua alta plasticidade em se desenvolver e se manter em diferentes condições de solo e clima, e por estar ameaçada de extinção, necessita de tecnologias relacionadas a propagação e conservação do material genético para garantir sua existência. No entanto, a principal forma de propagação da espécie ocorre via sementes, que tem como limitação a baixa taxa de germinação, e por ser lenta e desigual no ambiente natural e no viveiro, principalmente devido à dormência tegumentar e fisiológica da semente, e contaminação fúngica. Uma alternativa para isso é a micropropagação *in vitro* de embriões zigóticos, que proporciona alta taxa de germinação, em larga escala em ambiente asséptico. A germinação é fortemente influenciada pelo genótipo, pois está associada à expressão de características morfofisiológicas das sementes e anatômicas foliares. Dentre as iniciativas de melhoramento genético da espécie, foram gerados alguns genótipos, dos quais se destacam o 606 e o Santa Fé, o primeiro se destacando por suas características de crescimento da matriz, e o segundo pela produtividade de sementes e frutos. Portanto, este trabalho busca identificar marcadores morfofisiológicos e anatômicos que diferenciem diferentes genótipos de castanheiras, bem como investigar métodos de propagação mais eficientes para a produção de mudas da espécie. Para isso, sementes dos dois genótipos foram coletadas no estado do Amazonas, e de um genótipo selvagem no estado do Pará, que tiveram seus tegumentos removidos, e então foram levadas ao Laboratório de Fisiologia e Bioquímica Vegetal, onde foram mensuradas suas variáveis morfofisiológicas , semeadas em vermiculita expandida, e os embriões zigóticos dos genótipos 606 e selvagem foram inoculados em meio MS completo (Murashige Skoog); após a plântula completa, foi descrita a morfoanatomia da folha. Quanto à morfofisiologia, todos os genótipos apresentaram diferenças significativas, sendo também detectadas diferenças morfoanatômicas nas folhas das plântulas. O genótipo 606 se destacou quanto ao tamanho da semente, taxa de germinação e parâmetros que envolvem a velocidade de germinação. Com essas variáveis, foi possível agrupar os dois genótipos em uma análise de componentes principais (PCA), onde a variável mais associada a Santa Fé foi a quantidade relativa de água absorvida, enquanto para o genótipo 606 foi a porcentagem de germinação. Quanto às características morfoanatômicas das folhas das plântulas, houve diferença na morfologia entre o genótipo selvagem e os genótipos cultivados. Esses resultados mostram diferenças significativas entre os genótipos, facilitando a diferenciação entre eles, e permitindo escolher, dentro das condições deste estudo, o genótipo 606 como o mais adequado para produzir sementes e mudas de qualidade. A micropropagação in vitro mostrou-se promissora em relação à diminuição do tempo de germinação e formação de plântulas, onde houve um decréscimo de 74 dias para formação de plântula, para o genótipo selvagem, e 161 dias para o 606, quando comparado em cultivo vivo e in vitro. A morfologia da germinação entre os genótipos e as formas de propagação foi diferente, pois os embriões zigóticos apresentaram coloração verde mais intensa, enquanto no cultivo *in vivo* apenas algumas porções das sementes ficaram verdes e, além disso, alguns explantes selvagens não desenvolveram raízes, somente parte aérea. Portanto, para a castanheira do Brasil, é possível utilizar os marcadores relacionados à biometria e germinação da semente, bem como a morfologia da lâmina foliar para identificar o genótipo selvagem, o genótipo cultivado e, além disso, a micropropagação in vitro provou ser um método promissor para a produção de mudas de castanha-do-brasil em tempo inferior ao utilizado na propagação via sementes in vivo.

Palavras-chave: Castanheira do Brasil, cultura de tecidos, curva de embebição, tecnologia de sementes, produção de mudas

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Chapter 1: Deciphering the role of the morphophysiology of germination and leaves morphoanatomy for differentiation of Brazil nut genotypes

Chapter 2: Comparative study of the propagation process and the anatomical structure of the leaf of different genotypes of *Bertollethia excelsa* in two cultivation environments

1. GENERAL INTRODUCTION

In recent years, the growing and urgent need for the recovery of degraded areas and restoration of the landscape has led to an increase in interest in the propagation of native forest species, since they meet these needs (Nunes *et al*., 2020; Mariscal *et al*., 2023; Zhang *et al*., 2023). From this perspective, research that enhances forest regeneration is of paramount importance, and studies involving seed technologies are a foundation for this definition of techniques and models (Candiani, 2006; Miranda *et al*., 2017; Dionisio *et al*., 2019a; Šerá, 2021).

Among the forest species native to the Amazon with potential for restoring degraded areas is the Brazil nut tree (*Bertholletia excelsa* Bonpl.) (Costa *et al*., 2022; Souza *et al*., 2023a; Fortes *et al*., 2023). The propagation of this species occurs mainly via seeds (Nascimento *et al*., 2010). These seeds are characterized by constituting the testa and woody tegmen as protection of the almond, which give it a physical dormancy (Auca *et al*., 2018). Furthermore, the seeds are recalcitrant and it is speculated that they also exhibit physiological dormancy (Camargo *et al*., 2000). In relation, the almonds, which are the edible parts of the chestnut, have a triangular shape and there is no morphological differentiation in relation to the cotyledons, plumule and the hypocotyl-radicle axis (Baskin and Baskin, 2014; Dionisio *et al*., 2019). Brazil nut tree germination is slow and uneven, and often with low percentages, even under favorable conditions (Auca *et al*., 2018).

Due to the inefficiency related to the germination of the species, some studies indicate the importance of using technologies that can circumvent the problem of the low rate and irregular germination of the chestnut tree (Santos *et al*., 2013). For this, in vitro micropropagation is an alternative to the conventional production of seedlings, since it allows the mass propagation of the species, with a high germination rate and shorter seedling formation time, in addition to being an efficient way of preserving the germplasm (Pawłowski and Staszak 2016; Sánchez 2022).

Among the factors that influence seed germination, the genetic constitution of organisms, that is, the genotype, can be highlighted. Genotypes are strongly influenced by maternal characteristics, which are imprinted in the seeds, and result in the phenotypic plasticity and morphology of their progenies (Donohue, 2009; Donohue *et al*., 2010; Nguyen *et al*. 2021). Therefore, genetic improvement makes it possible to select genotypes for the production of

seeds and seedlings that allow for greater uniformity in the quality of seeds and seedlings produced (Geshnizjan, *et al*., 2019).

At Fazenda Agropecuária Aruanã, where there is the largest chestnut tree restoration plantation in the world (Ferreira, 2013), there are six Brazil nut tree genotypes, which are clones Santa Fé I, Santa Fé, II, C-606, C 609 and Manuel Pedro. These genotypes vary in fruit and seed productivity, where the genotype Santa Fé stands out due to the measures of growth of the matrices, mainly in relation to crown diameter and stem height, while the genotype 606 can stand out due to the high productivity of seeds and fruits, in addition to being larger, despite of the biometry of adult plants being the smallest of all genotypes. (Passos *et al*., 2018). In addition to productivity, there is a difference in photosynthetic productivity, and biometry of seeds and fruits (Ferreira, 2013; Passos, 2018). However, there are no reports in the literature about the germination characteristics of these genotypes.

Knowledge about the characteristics of the germination process of these different genotypes from the perspective of the morphophysiology of seeds and morphoanatomy of the leaf, as well as the exploration of biotechnologies related to the culture of plant tissues, can contribute to the advancement of clonal silviculture of Brazil nut trees, enabling greater control over the production process of seeds and seedlings of this species., with the purpose of applying this knowledge in planting management, with the objective of recovering degraded areas (Camargo, 2000; Silva *et al*., 2014). Therefore, it is necessary to obtain basic information about germination and the processes that involve it, as well as the investigation of in vitro plant micropropagation protocols, with the aim of discovering the potential of native species, in order to envision their uses for the most different purposes, since knowledge about production technologies involving native forest species is still incipient, especially when considering the analysis of these seeds (Ribeiro-Oliveira and Ranal, 2014).

2. THEORETICAL REFERENCE

2.1 Botanical characterization

The Brazil nut tree, also known as Castanheira-do-Pará and Castanheira-da-Amazônia, is considered the only species of the genus *Bertholletia*, and despite notoriously presenting morphological differences in fruit size and number of seeds per fruit, it was not necessary to recognize more than one species (Mori 2014; Costa *et al*, 2022). The species was described by Poiteau in 1825, however, it was first characterized by the French botanist Aimé Jacques

Alexandre Goujaud Bonpland in volume 1 of his work entitled “Plantes Équinoxiales” (Mori, 2014; Mori, 2018; Costa *et al*., 2022). The name of the Genus is in honor of the French chemist and physiologist Claude Louis Berthollet, while the specific epithet “*excelsa*” has the majestic meaning, high, in reference to the tall size of the plant (Mori, 2018; Costa *et al*., 2022).

Bertholletia. excelsa Bonpl. can reach 30 to 60 meters in height, with the diameter of the base exceeding 4 meters. Its trunk is straight, cylindrical and without branches, its diameter at breast height – (DBH) varies from 100 to 180 centimeters (Muller *et al*., 1995; Lorenzi, 2008; Salomão, 2009; Carvalho, 2014; Sconghart, *et al*., 2015; Costa *et al*., 2022). The trunk has branches with curved branches at its ends. Its leaves are alternate, thick and pale green on the abaxial part and dark green on the adaxial part; leathery blade with wavy margins and acute base, its shape is like a trough and its dimensions vary from 8 to 12 cm in width and 25 to 35 cm in length, the petioles that support them measure from 5 to 6 centimeters in length. (CNI *et al*., 2004; Carvalho, 2014).

Its reproductive organ is a panicle-type inflorescence, with axes formed by spikes, and zygomorphic floral constitution. Its flowers have six large whitish petals, with a concave and deciduous shape; the ovary is covered, so its pollination is carried out only by bees of the genus *Bombus*, *Centris*, *Epicharis*, *Eulaema* and *Xylocopa*, as they are the only ones with the strength to enter the flowers. The style is superior to the anthers; the flowers still develop in straight and vertical panicles, with racemose ends (Moritz, 1984; Cymerys *et al*., 2005; Carvalho, 2014; Maués, 2015).

The fruit of the Brazil nut tree is commonly known as “ouriço”, with the shape of a pixid-type caryopsis, weighing from 200 g to 1.5 kg; with a diameter between 8 and 16 cm, and covered by a woody integument. In its inner part are located the seeds, varying from 10 to 25 in each hedgehog, which are interconnected by a woody and dehydrated membrane, similar to orange segments, weighing between 4 to 10g, with an angular triangular shape, and are also covered by a woody tegument divided into testa and tegmen (bitegumentate), which protects the embryo (Santos *et al*., 2006; Petrechen, 2017; Petrechen *et al*., 2019).

2.2 Ecology and geographical distribution

Bertholletia. excelsa Bonpl. is luciferous and helioferous, for a plant strongly linked to sunlight. In the first years, growth in height is prioritized, and when it reaches the forest canopy, the source of light energy in abundance found there, provides a greater investment by the

species in growth in diameter (Salomão, 1991; Tonini *et al*., 2014). Tonini *et al*. (2014), state that when in the presence of abundant light intensity, the chestnut tree has its initial growth accelerated, whereas Kainer *et al*., (2014) argue that the opposite also occurs when light availability is low.

The Brazil nut tree is considered a sociable species, due to its occurrence in certain regions it is easily found in clusters in the forest composition (Costa *et al*., 2022).

The species is geographically distributed throughout the Amazon region, including countries such as Brazil, Venezuela, Colombia, Peru, Bolivia and the Guianas. In Brazil, it is found in the states of Amazonas, Roraima, Acre, Pará, Rondônia, Amapá and the north of Mato Grosso. Precipitation in these mentioned regions ranges from 1400 to 2800 mm per year, while relative air humidity is around 79 to 86%. Furthermore, the average annual temperature fluctuates between 24.3º and 27.º C; with maximum and minimum values of 30.6º and 32.6ºC, and 19.2º and 23.4ºC respectively. These climate characteristics provide the best growth and development for the species, and the wide variation in these variables demonstrates the plasticity of the species under different conditions (Diniz and Basto, 1974; Guerreiro, 2017; Costa *et al*., 2022).

The dissemination of the Brazil nut tree occurs mainly through wild animals, mainly rodents such as the agouti, which bury the seeds in places far from their matrices, at a depth of 1 to 3 cm, which are forgotten and germinate after 12 to 18 months (Peres and Baider, 1997; Mori and Prance, 1990; Haugaasen *et al*., 2010; Costa *et al*., 2022; Souza *et al*., 2023a).

2.3 Genotypes of Brazil nut tree

In 1968, research related to the genetic improvement of the *Bertholletia excelsa* Bonpl. via grafting began at Embrapa Amazônia Oriental, in the municipality of Belém-PA. Twelve parent trees distributed between the municipalities of Alenquer and Oriximiná, with different heights and stem diameters were selected (Table 3) (Muller et al. 1995; Nascimento et al. 2010; Nascimento et al., 2023).

These genotypes were transported to Empresa Agropecuária Aruanã in the municipality of Itacoatiara - AM (Table 4), where they were planted in an area that was previously destined for pasture, and became one of the main commercial plantations of Brazil nuts fruits and seeds in the Brazil in the Amazon (Müller, 1982; Mori, 1992).

Table 1. Origin of genotypes of *Bertholletia excelsa* from Aruanã Farming Company S.A., located in the municipality of Itacoatiara – AM.

Origin	Subject	Genotype	Shaft heigh of matrices (m)	Diameter of the matrices stem (m)
Castanhal Santa Fé, on the banks of the Mamiá River, Alenquer-PA.	PA1, PA4 e PA8	Santa Fé I	27	5,35
Castanhal Santa Fé, on the banks of the Mamiá River, Alenquer-PA.	PA14, PA15, PA16	Santa Fé II	24	4
Castanhal Porongaba, on the banks of the Mamiá River, Alenquer-PA.	-	Manoel Pedro I	-	-
Castanhal Segredo, on the banks of the Mamiá River, Alenquer-PA.	PA5, PA9, PA13	Manoel Pedro II	-	-
Airport road, city of Alenquer-PA.	PA3, PA10, PA12	606	6	1,43

Source: Adapted from Serra *et al.*, (2006); Nascimento *et al*. (2010), (Passos, 2014), Nascimento *et al*., (2023).

After the establishment of the matrices in Aruanã, at the age of 31 years, Manoel Pedro and Santa Fé stood out in relation to the parameters Diameter of Breast Height (DBH), in contrast, the C-606 had a statistically inferior performance (Ferreira, 2013). The Manoel Pedro genotype also produced more fruits and seeds, while the C-606 and Santa Fé produced less when compared to Manoel Pedro, on the other hand, the Santa Fé genotype showed higher seed and fruit productivity when compared to the 606, and higher DBH, as well as its correlation with productivity and biometry of fruits and seeds (Passos, 2018) (Table 2).

Table 2. Biometrics mensurements of matrices of genotype of *Bertholletia excelsa,* from Aruanã Farming, located in municipaly of Itacoatiara - Amazonas state.

Genotype	High (m)	DBH (cm)	Canopy area (m^2)
Santa Fé	23.5 ± 4.5	75 ± 13	378 ± 132
Manuel Pedro	20.5 ± 4.0	88 ± 10	293 ± 66
606	16.0 ± 3.0	58 ± 19	243 ± 88

Source: Adapted from Passos (2014).

2.4 Morphoanatomy of seeds and seedlings of *Bertholletia excelsa*

2.4.1 Seeds

In general, the seeds of angiosperms have similar structures. They are covered by a protective layer made up of dead cells called integument (Taiz *et al*., 2017). The integument protects the embryo from the seed, which consists of the embryonic axis and a cotyledon, in the case of species belonging to the class Liliopsida, or two cotyledons, if it belongs to the class of magnoliopsids (Taiz *et al*., 2017). Cotyledons are reserve tissues and are degraded during germination (Rajjou, *et al*., 2012; Taiz *et al*., 2017; El-Maarouf-Bouteau, 2022). The embryonic axis is divided between the radicle, which will originate the root of the seedling; hypocotyl, where the cotyledons are adhered, and the cauline axis, bearing the plumule, or the leaf primordia (Taiz *et al*., 2017). The embryo may be surrounded by another reserve tissue called the endosperm, usually consisting of proteins, carbohydrates, and lipids, and when it is present, the seeds are classified as endosperm, and when they are absent, they are classified as non-endosperm (Taiz *et al*., 2017).

The seeds of *Bertholletia. excelsa* Bonpl. are bitegmic, consisting of the testa, which is the external integument, highly lignified, and the tegmen, which is the inner layer (Figure 1). The testa has a waxy and wrinkled surface, with a brown color of different intensities, comprises three faces that are connected together and that connect at the two ends of the seeds, which give them the triangular shape (Scussel *et al*., 2014). Still on the forehead, it is possible to observe a locule that is also called micropyle (Scussel *et al*., 2014). The tegmen, which is the part of the bark that is brown in color and is attached to both the testa and the edible part, is formed by flattened, irregular parenchymal cells distributed in several layers with fibrous wall tissue that is more flexible and thinner than the bark and has a smooth texture. rough and shiny smooth (Scussel *et al*., 2014).

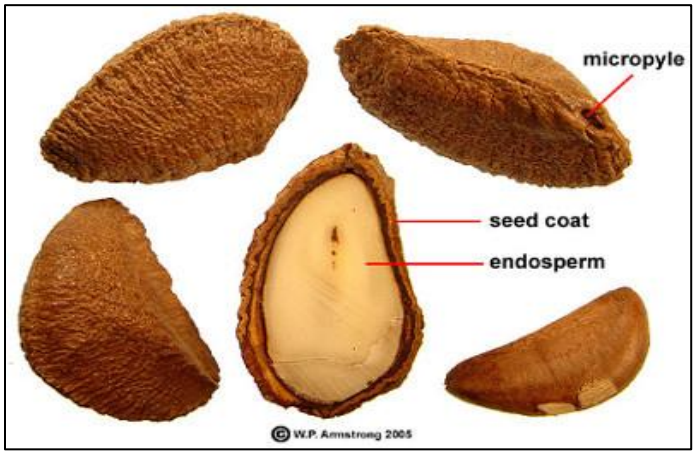

Figure 1. Morphology of seeds of *Bertholletia excelsa.*

The outermost region is the endosperm, which in the case of chestnut seeds assumes the function of a protective tissue, consisting of an epidermis layer 40 to 50 μm wide (Scussel *et al*., 2014). Adjacent to the epidermis, there are layers of parenchyma cells filled with proteins and lipids that constitute the reserve storage tissue, and make up most of the edible tissue of the chestnut, however, there is no differentiation of the cotyledons, nor of the hypocotyl-radicle axis (Santos *et al*., 2006; Scussel *et al*., 2014). Between the layers of parenchyma cells there is a small ring of meristematic tissue, which can originate other tissues when stimulated by growth regulators (Camargo, 2000; Scussel *et al*., 2014). Camargo (2000) describes the chestnut tree embryo as hypocotyl, because the root and aerial part originate from pre-existing meristematic tissues, making it assume the role of hypocotyl. Regarding color, the main tissue has a white to creamy light color and a slightly darker center and its width and length vary according to the size and shape of the chestnut.

Nuts in shell are classified according to the relationship between length and mass in the following categories: type I, II and III nuts, which correspond to large, medium and small nuts (Mello and Scussel 2007). Large chestnuts are longer than 50 mm and weigh from 6 to 18 g; medium nuts have a length ranging from 40 to 50 mm and a mass from 2 to 17 g, while small nuts have a length of less than 40 mm and a mass ranging from 1 to 11.3 g (Mello and Scussel 2007).

The morphometric characteristics of the seeds of the Brazil nut tree are characterized by having a great phenotypic variability that vary according to several factors, among which stand out the genotype, geographic location (Table 1) and edaphoclimatic conditions (Passos *et al*., 2018). The seeds produced by chestnut trees in the Solimões region, for example, stand out for being large. On the Purus River, there is a place called Abufari, where chestnuts reach 12 and 15 cm in length (Mello, 2000).

Table 3. Biometrical characteristics of seeds of *Bertholletia excelsa* with tegument.

Biometrics of *Bertholletia excelsa* seeds in different states of Brazil				
Origin	**Lenght (mm)**	**Width (mm)**	**Thickness (mm)**	**Mass (g)**
Mato Grosso[1]	40,09	26,31	17,43	3,28
Pará[2]	48,8	23,17	23,11	7,3
Amazonas[3]	47,2	28,7	21,2	*

1 Botelho *et al*., (2019); 2 Soares (2019); 3 Aguiar *et al*., (2018)

2.4.2 Seedlings

Brazil tree germination is characterized as cryptocotyledonary, hypogeal and with straight emergence. Germination time varies from 30 to 360 days, the radicle, in cross-section, is rounded, yellowish white, crassa, glabrous, measuring 5.21 x 1.09 mm (Santos *et al*., 2006; Dionisio *et al*., 2019a; Dionísio *et al*., 2019b). After 30 days, the cap and collar are imperceptible and the presence of alternating cataphylls is observed throughout the length of the epicotyl (Figure 3).

The root system of the seedling can be described as embryonic, the main root has a cylindrical shape, where the root grows, it thickens, resulting in a thick base and thin apex; it is brown in color, with a subwoody and glabrous texture; and little occurrence of secondary roots, which are filiform in shape, brown in color and subligneous and glabrous textures (Figure 2n) (Santos *et al*., 2006).

The neck, hypocotyl and cotyledons are inconspicuous. Epicotyl is classified as epigeous, and has a cylindrical shape, originating from the apex of the seed, its base has brown tones with many burst lenticels, from the median region to the apex a purplish brown color (Figure 2s) (Santos *et al*., 2006).

The cataphylls are triangular, purplish, alternate and pilose. Eophyllum one simple, obovoid, with involute prefoliation and brochidodromous veining, with prominent veins on the adaxial surface and imprinted on the abaxial surface. The apex is acute, with a slightly serrated edge in reddish brown tones, and its base is symmetrical and attenuated. The petiole is slightly canaliculate and subsessile, with no pulvinus. The second eophyll is similar to the first, with its phyllotaxis alternating in relation to it (Figure 2s) (Santos *et al*., 2006).

The formation of the shoot and root of the seedling comes from meristematic tissues located at the cauline and root poles, respectively (Camargo *et al*., 2000). The seedling root does not have a cuticle or thick cell wall, to facilitate the absorption of water and nutrients, in addition to having aerenchyma, intracellular spaces found in species with humid or arid climates (Camargo *et al*., 2000). The stem presents simple and unicellular trichomes and lenticels in the epidermal tissue. The leaves have unicellular trichomes, mainly on the abaxial surface, the thin cuticle, and their vascular bundles are more developed in the midrib.

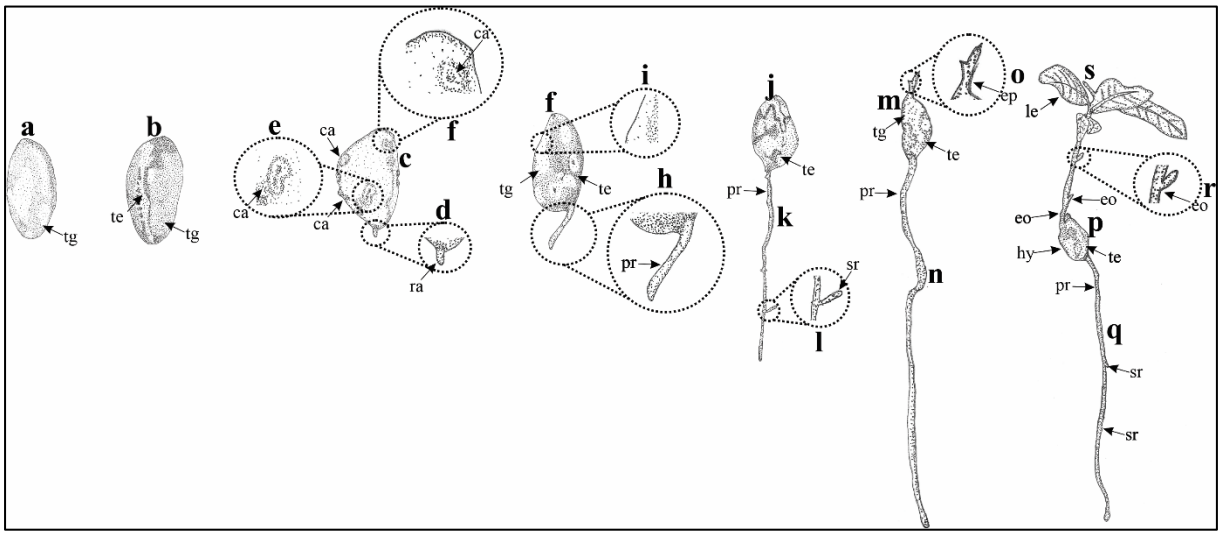

Figure 2. Morphology of germination of *Bertholletia excelsa*; a = quiescent seed; b = imbibed seed; c = radicle protrusion from seed; d = radicle; e = seed callus; f = callus and greenish coloring; g = radicle elongation from seed; h = root 1.5mm; i = greenish coloring j = secondary root from seed; k = pri-mary root; l = secondary root; m = phase six seed (epicotyl emission); n = primary root; o = epicotyl; p = eophylls from seed; q = primary root; r = (eophylls); s = leavesca = callus; eo = eophile; ep = epicotyl; pr = primary root; ra = radicle; sr = secondary root; te = testa; tg = tegmen.

2.5 Brazil nut seed germination

From a technological or agronomic point of view, the concept of germination refers to an intensification of seed metabolism and development of parts of the embryo, originating a seedling that presents all the structures originating from the hypocotyl-radicle axis, such as roots, epicotyl and eophiles (Brasil 2009; Martinez 2011). While, from a botanical or physiological point of view, germination begins with the resumption of metabolic activity, culminating in the elongation of the embryonic axis (most often the root axis), which characterizes the end of germination.

Germination is the first phase of a plant's growth and development, and can be decisive for its life cycle. In this phase, the form and functional characteristics of higher plants are assumed, that is, the structure and functioning of adult individuals are defined from germination onwards (Fu *et al.*, 2005). This phenomenon incorporates events that begin with the absorption of water (imbibition) by the quiescent seed, or overcoming dormancy and results in the expansion of the embryonic axis, with the emission of the radicle. In these events, intense metabolic activity occurs, resulting in structural changes in the seeds (Bewley 2001; Borghetti *et al.*, 2004; Bewley *et al.*, 2013; El-Maarouf-Bouteau, 2022), as shown in Figure 3.

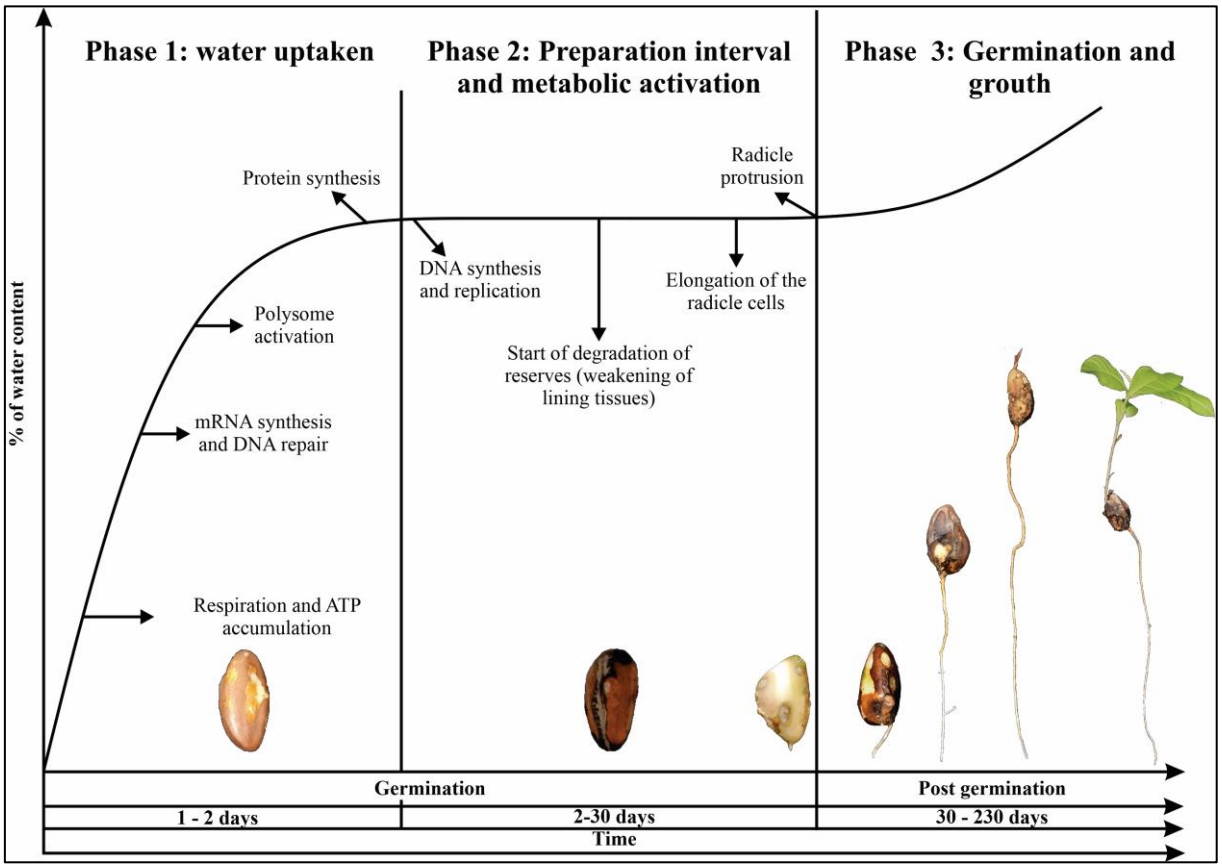

Figure 3. Imbibition curve on germination**.**

Germination can be evaluated by determining the germination rate (%G) which represents the percentage of germinated seeds in relation to the number of seeds placed to germinate; mean germination time (MGT), which quantifies germination from a kinetic point of view, that is, it informs the time required for a given sample of seeds to germinate (Maguire, 1962; Labouriau and Valadares, 1976; Carvalho and Nakagawa, 2000; Talská *et al*., 2020). The variation in the mean germination time reflects its temporal distribution around the mean, which allows assessing whether the germination of a given set of seeds is uniform (small variation) or uneven and irregular (large variation) (Carvalho and Nakagawa, 2000; Silva *et al*., 2010). Another way to evaluate the germination kinetics is by determining the coefficient of velocity of germination (CVG) in which the number of seeds or normal seedlings is counted each day and the higher the CVG, the greater the germination speed, which allows inferring the more vigorous the seed lot is (Carvalho and Nakagawa, 2000; Talská *et al*., 2020).

The germination of *B. excelsa* seeds is slow and uneven, and often with low percentages, even under favorable conditions (Auca *et al*., 2018; Dionisio *et al*., 2019b). However, without the seed coat and in forest soils, it is possible to observe germination of 81% at 30 days, but irregularly (Auca *et al*., 2018). This slow germination process may be associated with the existence of chemical dormancy (presence of inhibitors) and/or morphophysiological dormancy (embryo immaturity) (Baskin and Baskin, 2004; Buckeridge *et al*., 2004; Ribeiro *et al*., 2016; Kildisheva *et al*., 2016; Rodrigues-Junior *et al*., 2018). The lack of uniformity and the long period of germination of the chestnut tree may be associated with the need for time for the process of imbibition, enzymatic activation, and differentiation of the existing meristematic tissues in the almond to occur. This behavior can also be affected by the large number of reserves in the embryo and/or by an internal hormonal balance (Camargo *et al*., 2000).

During the germination process, the mobilization of reserves is triggered and extends to the formation of seedlings (Raven *et al*., 2007). Thus, when seed germination begins, protein synthesis resumes and, consequently, the formation of hydrolytic enzymes responsible for mobilizing reserves (Dantas *et al*., 2008). Carbohydrates, proteins, and lipids are mobilized during germination and, during seedling development, their degradation products are used for different purposes, such as energy generation and the production of raw material for building new cells and tissues (Mayer and Poljakoff-Mayber, 1975).

In addition to macromolecules (carbohydrates, lipids, and proteins), nutrients also influence the chemical composition of seeds and, consequently, their metabolism and vigor.

Minerals are deposited in the form of phytin (myo-inositol hexaphosphate), where phosphorus, calcium and magnesium ions are stored. These ions play important roles in germination metabolism, and the embryonic axis needs this source until the root is developed enough to extract them from the substrate (Buckeridge *et al*., 2004; Titok *et al*., 2015). Still about mineral prospecting, phosphorus is perhaps the nutrient that plays a fundamental role in the life cycle of plants, participating in metabolic processes, energy transfer, the initial phase of the reproductive parts, root development and fruit and seed formation (Peske *et al*., 2009).

2.6 Effect of genotype on germination

Among the factors that influence seed germination, internal factors such as vigor, desiccation tolerance, dormancy and genotype can be highlighted, and external factors that include the availability of water, oxygen, light, temperature, substrate, and nitrate. These factors can influence germination both by acting on the speed of water absorption and by the intensity of the biochemical reactions that determine the process (Carvalho and Nakagawa 2012). When seeds are exposed to the same environmental condition, internal factors begin to determine the germination capacity of the seed. Among the internal factors, the genotype assumes a prominent role, since it conditions the other internal factors that can influence germination (Ramos *et al*., 2011; Nascimento *et al*., 2016; Lasky *et al*., 2023).

The genotype can be defined as the genetic composition or the set of alleles of an organism (Lasky et al., 2023). Seeds of different genotypes of the same species have different physiological and biochemical characteristics that can result in changes in germination capacity and seedling production (Wang *et al*., 2007).

2.7 Brazil nut propagation methods

The Brazil nut tree is propagated mainly sexually, however, genetic improvement programs for the species already address research related to grafting and micropropagation (Santos *et al*., 2013; Conceição *et al*., 2020, Souza *et al*., 2023b).

Despite being the most common, propagation via seeds has limitations, which are related to the physical and physiological dormancy of the seed, as well as its recalcitrance, making long-term seed storage unfeasible, and in an environment with low humidity (Souza et al. al., 2023b), in addition, the seed is used in human food, being an important source of

selenium, restricting its availability to produce seedlings sexually (Silva Junior *et al*., 2022; Souza *et al*., 2023b).

In order to promote genotypes resistant to pests and diseases and also to increase productivity and fruit collection, the technique of grafting was used in the species, which consists of using a rootstock from a wild matrix, which is generally more tolerant to pests. and diseases, with selected grafts that present desirable characteristics, such as high fruit productivity, lower stem height and earlier production (Carvalho *et al*., 2016; Tsaballa *et al*., 2021; Myers *et al*., 2023; Nascimento *et al*., 2021; Nascimento *et al*., 2023b). However, the practice of grafting still depends on the production of seedlings via seed, for the formation of rootstocks (Conceição *et al*., 2020).

Aiming to promote the genetic improvement of the species, and to use reproductive or vegetative organs for this purpose, some protocols related to the micropropagation of Brazil nut trees were established, through the in vitro cultivation of plant tissues, which use explants as propagules, which can be tissue vegetative, such as leaves, roots, and apical and axillary buds; as well as reproductive tissues, such as zygotic embryos, anthers, among others, however, the asepsis protocols for the species were not efficient to control contamination of the medium and explants. (Santos *et al*., 2013; Abdalla *et al*., 2022). Some plant tissues have cellular pluripotency or totipotency, which allow them to dedifferentiate their already differentiated cells, and return them to an "initial" state, which, from then on, through hormonal stimuli, can generate other tissues and specialized cells (Morinaka *et al*., 2023).

This technique consists of inoculating these explants in a culture medium containing sources of carbohydrates, vitamins, minerals, gelling agents, water, and in some cases, growth regulators and microbiocides to control fungal and bacterial contamination (Santos *et al*., 2013; Abdalla *et al*., 2022; Coelho and Romano, 2022). This allows an aseptic environment in the cultivation of these explants, in addition to allowing the mass propagation of the species from little vegetative material, facilitating the genetic improvement of plant species, including the Brazil nut tree. When it comes to *B. excelsa*, there is a lot of contamination by endophytic fungi, both in vivo and in vitro cultivation (Santos *et al*., 2013).

The latter is very affected by fungal contamination when in its initial stage of development, especially during germination, which can be mitigated through in vitro cultivation (Santos et al., 2013), and in relation to the economic importance of its seeds, its use could be restricted, and largely replaced by leaf explants, due to the high concentration of meristematic cells (Camargo, 2000).

Despite the potential of plant tissue culture in the micropropagation of the species, there are few related works, mainly due to the difficulty of adapting efficient asepsis protocols.

3. OBJECTIVES

General objetives

- To investigate the morphophysiological, anatomical and propagative aspects of seeds and seedlings during germination and seedling formation of different genotypes of *Bertholletia excelsa* Bonpl.

Specific objectives

- To describe the morphophysiological characteristics of seeds from different genotypes of *B. excelsa*.
- To describe and characterize the morphoanatomy of leaves from seedlings of different genotypes of *B. excelsa*.
- To describe and characterize the propagation methods of the different genotypes of *B. excelsa*.
- To describe and characterize the vegetative structures of seedlings of different genotypes of *B. excelsa*, formed under different conditions.

4. HYPHOTESIS

- ☐ Is it possible to classify and select superior genotypes, based on morphophysiological characteristics of seeds and morphoanatomical characteristics of leaves, to produce quality Brazil nut seedlings?
- ☐ Based on morphophysiological and anatomical characteristics of seeds and seedlings, is it possible to identify markers for identification of *Bertholletia excelsa* genotypes?
- ☐ Using the different propagation methods, is it possible to produce quality seedlings free of phytopathogens and production in large quantities?
- ☐ Are there differences in the vegetative structures of seedlings propagated ex vitro and in vitro?

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Chapter 1: Deciphering the role of the morphophysiology of germination and leaves morphoanatomy for differentiation of Brazil nut genotypes[1]

[1] This manuscript has been submitted to the Brazilian Journal of Botany.

Abstract: The Brazil nut tree (*Bertholletia excelsa* Bonpl.), one of the main species from the socioeconomic and ecological point of view of the Amazon, is characterized by slow and uneven germination. Considering that its genetic constitution is a determining factor for the morphology and physiology of germination, we investigated different Brazil nut tree genotypes in regards to morphophysiological seed traits and performed a morphoanatomical description of the leaves. Genotypes showed differences in seed morphology, imbibition curve, germination rate, and germination speed index, and also, in some of the morphoanatomical leaf structures. The genotype 606 showed the best physiological performance during germination, mainly in terms of water imbibition. Our findings aid the morphoanatomical differentiation of different genotypes of *B. excelsa*, and represent a significant step forward for clonal propagation, as well as providing a route for improving these species physiological and genetic characteristics.

Keywords: *Bertholletia excelsa*, imbibition curve, seed technology, seedling production, leaf traits

1 Introduction

In recent years, the interest in propagating native forest species for the recovery of degraded areas has increased, especially in the Amazonian region (Nunes et al. 2020; Mariscal et al. 2022; De Souza et al. 2023; Zhang et al. 2023). From this perspective, research that enhances forest regeneration is of paramount importance, and studies involving seed technologies are a foundation for defining techniques and plantation models (Candiani 2006; Ribeiro-Oliveira and Ranal 2014; Mousavijad et al. 2023).

Among the forest species native to the Amazon that have the potential for restoration/recovery of degraded areas is *Bertholletia excelsa* Bonpl. (Costa et al. 2022; Fortes et al 2023; Zhang et al 2023). Belonging to the Lecythidaceae family, and popularly known as the Brazil nut tree, it is propagated mainly by seeds (the use of seedlings from seeds is important for wood production), and asexually by grafting (the use of rootstocks is recommended for the production of Brazil nut fruits). However, grafting can also be considered an effective method for the genetic improvement of timber species, mainly to express productivity characteristics and desirable robustness for wood, on the other hand, for Brazil nut fruit production this method is very important in reducing the size of the plants. (Nascimento et al. 2010; Tsaballa et al. 2021; Myers et al. 2023; Nascimento et al. 2023; Santos et al. 2023; Sanogo et al. 2023).

In 1968, began the genetic improvement of Brazil nut trees, using matrices with different heights and stem diameters, from the municipalities of Alenquer and Oriximiná, in the state of Pará. This improvement had as its main objective the enhancement of fruit production by the species, where from these selected matrices, cuttings were removed and grafted onto rootstocks with more resistant and robust matrices. From the municipality of Alenquer, three matrices with stem height measuring 24 and three with 27 meters, and stem diameters measuring 5.35 and 4 meters were selected of which the genotype Santa Fé was originated. In another area of the same municipality, three matrices were selected, each with a stem height measuring 6 meters and a stem diameter measuring 1.43 meters, generating genotype 606 (Ferreira 2013; Passos et al. 2018).

Of these genotypes, Santa Fé stands out with regard to the diameter at breast height (DBH), correlated with fruit and seed productivity; on the other hand, without taking correlation into account, this genotype has smaller fruits and seeds, in addition to lower seed productivity per fruit. Meanwhile, the genotype 606 produces larger fruits, with more seeds, and these are also larger, when compared to Santa Fé, despite having lower biometric characteristics than the mother plants, also when compared to the genotype Santa Fé, therefore, these genotypes fit into two distinct and opposite parameters in terms of desirable characteristics for the production of quality seeds and seedlings (Passos et al. 2018). However, research related to the morphophysiological factors associated with the germination, growth, and development of seedlings of different genotypes of *B. excelsa* is still scarce.

When it comes to seed morphology, the Brazil nut tree has a woody and bitegmic integument, consisting of the testa and tegmen that give it physical dormancy (Auca et al. 2018). Furthermore, the seeds are recalcitrant and it is speculated that they also exhibit physiological dormancy (Camargo et al. 2000). The nuts have a triangular shape, without morphological differentiation in relation to the cotyledons, plumule, and the hypocotyl-radicle axis. The chemical composition is characterized by a high content of fixed oils (lipids), good quality proteins for the food industry (Silva et al. 2009), and selenium (Lima et al. 2019; Silva Junior et al. 2022).

Due to its physiological and biochemical characteristics, the germination process of the Brazil nut tree is slow and uneven, and often presents low percentages of germination/emergence, even when under favorable conditions (Giustina et al. 2017; Auca et al. 2018). From a physiological point of view, germination begins with the resumption of metabolic activity shortly after the absorption of water by the seed and culminates in the elongation of the embryonic axis, which is generally divided into three phases (Rajjou et al. 2012; Taiz et al. 2017; El-Maarouf-Bouteau 2022). In the first phase, the imbibition phase, rapid water absorption occurs, which activates the seed's metabolism (respiration, transcription, and translation); transition from the paracrystalline state of the cell membrane to the liquid state; and causes changes in gene expression. In phase II, the mobilization of the seed's primary reserves (proteins, carbohydrates, and lipids) occurs, which ends with the emission of the radicle (Rajjou et al. 2012; Taiz et al. 2017; El-Maarouf-Bouteau 2022). In phase III, there is elongation of the radicle, degradation, and mobilization of primary reserves in a more complex manner; cell division, seedling growth, and development (Rajjou et al. 2012; Taiz et al. 2017; El-Maarouf-Bouteau 2022).

Seed morphology is one of the main factors that influence germination, and is influenced mainly by the genotype; therefore, it is an important parameter for the production of seeds and seedlings (Costa et al. 2006; Silva et al. 2010; Geshnizjan; 2019; Duarte et al. 2021; Ferraz et al. 2021). Knowledge of the characteristics of the germination process of these different genotypes contributes to a more practical control over the production process of seeds and seedlings of this species. This is related to the processes that involve seed germination, since it is strongly influenced by water absorption, reflecting on parameters related to germination, (e.g., percentage of protrusion and germination), and germination time (e.g., mean germination time, coefficient of velocity of germination, and mean of velocity of germination). In addition to the morphophysiology of the seed, the genotype also determines the morphoanatomical characteristics of the leaf, making it an important characteristic of genotypic differentiation. Therefore, elucidating the germination process of the species, in addition to the distinction between leaf morphoanatomical characteristics, directly implies as a management plan tool for the recovery of degraded areas, and thus, contributing to the advancement of Brazil nut clonal forestry (Camargo et al. 2000; Silva et al. 2014).

Considering the points described above, it is pertinent to raise the hypothesis that it is possible to classify and select superior genotypes to produce quality seeds and seedlings, based on morphophysiological characteristics of germination and morphoanatomy of leaves. As such, the objectives of this work were to investigate the morphophysiological aspects, during the germination and formation of seedlings of different genotypes of *B. excelsa*, as well as describe the morphophysiological characteristics of seeds and seedlings from different genotypes of *B. excelsa* throughout the germination and initial growth of seedlings from the seeds. The results obtained here demonstrate that not only seed related parameters but also that further indicators (morphoanatomy and physiological) can be used in the Brazil nut breeding program.

6. Materials and Methods

2.1 *Collection of plant material*

Seeds from two *Bertholletia excelsa* Bonpl. genotypes (Santa Fé = SF and 606) were collected from previously selected matrices, in the seed collection area of Aruanã Farming Company® S.A (3°0'30.63" S, 58°50'1.50" W), which is located in the municipality of Itacoatiara, Amazonas state, Brazil (Figure 1). The predominant soil in the seed collection area

is the Dystrophic Yellow Latosol with a very clayey texture (Projeto Radam Brasil 1978) and the climate in the region is of the Amw type, with an annual average temperature and precipitation of 27 °C and 2,300 mm year-1, respectively. Regarding the seeds of wild matrices (WS), these were collected in the municipality of Parauapebas, in the state of Pará, in a private rural property.

After collection, the seeds were placed in plastic bags and transported to the Laboratory of Plant Physiology and Biochemistry (National Institute for Amazonian Research - INPA), where they were processed and the experiments were carried out.

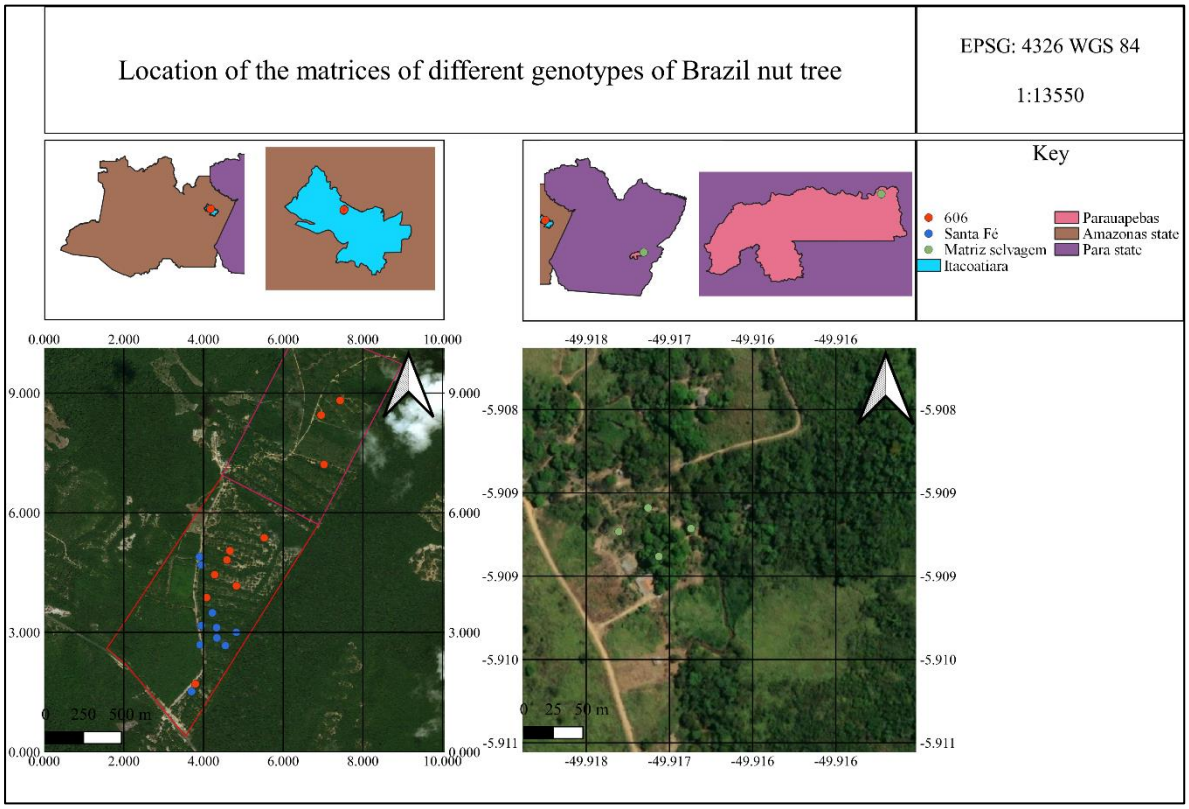

Fig. 1 Location map of the *Bertholletia excelsa* matrices

Fruit collection of the cultivated genotypes took place in February 2022, according to the dispersion period of the matrices, while the wild fruits occurred in January 2023. The collection was limited to the area covered by the canopy of the tree. The Aruanã'a fruits were taken to the storage house of Aruanã Farming Company, where they were opened with the aid of a machete and had their seeds extracted. The seeds of the three genotypes had their teguments removed with the aid of a machete, and were subsequently immersed in a solution of garlic vine leaf (*Mansoa aliacea* A.H. Gentry), and distilled water, for five minutes to perform the antifungal control (Shukla et al. 2008; Aswini et al. 2010), according to the protocol used at Aruanã Farm Company. After this time, they were dried at room temperature, for 12 hours, and then packed in semipermeable plastic bags for transport.

The treated seeds were taken to the Laboratory of Plant Physiology and Biochemistry, at the Nacional Institute of Amazon Researches, campus V8, where they were sown in vermiculite substrate of medium granulometry, autoclaved for 10 minutes at 1 Atm.

2.2 *Morphophysiological characterization*

2.2.1 Biometrics of seeds and fruits

The biometric determinations were carried out with the aid of a digital caliper, with a precision of 0.01 mm, measured in three manners: length, diameter and number of seeds, for the fruits; and length, width and thickness for the seeds. In the fruit measurements, length was considered as the longitudinal measurement between the apex and the base of the fruit, the diameter in the middle region, and the number of seeds present in each fruit. For the seeds, length was considered as the longitudinal measurement between the apex and the base of the seed, the width was the measurement of the median region transverse to the length, and thickness was the measurement of the median region parallel to the length. For this analysis, 20 fruits of each genotype and 100 seeds of each genotype were used.

Seed mass was measured using an analytical balance, with a precision of 0.001 g, and the seeds used were from the same lot as those used for the imbibition curve.

2.2.2 Imbibition curve

A total of 15 seeds were weighed right after the tegument was removed, and later soaked in distilled water. After imbibition, successive weighings were carried out at periodic intervals, starting at two hours immediately after the start of imbibition, then four hours, until completing 12 hours; henceforth, with intervals every 24 hours and, from then on, the weighings were carried out until the stabilization of the seed mass, as demonstrated in the adapted methodology found in Reis (1979).

2.2.3 Absolute and relative water content

For the same lot of seeds as used in the imbibition curve, an average of two seeds divided into eight repetitions were used to calculate the absolute and relative amount of absorbed water, as described in the following equations:

- Absolute Water Content (AWC)

$$AWC=fm-im$$

fm: final mass of water, after the last soaking time; im: seed initial mass.

- Relative Water Content (RWC)

$$RWC = \frac{fm}{im} \text{ X mi}$$

fm: final mass of water, after the last soaking time; im: seed initial mass.

2.3 *Germination Test*

The monitoring of the counting of germinated seeds occurred every three days. Data for three stages of germination were collected: Start of the protrusion (SP), defined as the moment the radicle broke the nut's tegmen, radicle protrusion (RP), defined when the radicle measured 3 mm in length; and radicle elongation (RE), when the radicle measured from 1.5 to 2 cm in length. For each treatment, the germination percentage (G%), mean germination time (MGT) according to Labouriau and Valadares (1976), coefficient of the velocity of germination (CVG),

mean velocity of germination (MVG) and percentage of root protrusion precocity (PRPP), adapted from the methodology of Maguire (1962).

Table 1 Description of equations for germination tests

Parameters	Symbol	Equation	Descriptions	Unit
Germination Percentage	%G	%G = (N/A). 100	N = number of seeds germinated; A= number of seeds sown	percentage
Mean Germination Time	MGT	MGT = (Σsgit) / Σsg	t = average incubation time; sg = number of seeds germinated per day; it = incubation time (days)	day
Coefficient of Velocity of Germination	CVG	CVG = Σ(sg/it)	sg = number of seeds germinated on it; it = incubation time	dimensionless
Mean of Velocity of Germination	MVG	MGV = 1/MGT	MGT = Mean Germination Time	day^{-1}
Root Protrusion Precocity Percentage	RPPP	RPPP = ΣSe/ΣIp	ΣSe = sum of the number of seeds that emitted the primary root (>1mm) per day; Ip = periods in days those emissions occurred	percentage

2.4 *Morphology of germination and initial growth of seedlings*

The morphological characterization of seeds was carried out using samples of 10 units from each stage of germination and seedling formation. Observations were performed with the aid of a Zeiss© table magnifying glass and a stereomicroscope.

2.4.1 Leaf anatomy

The leaves were detached from the stem during cultivation. This was followed by freehand transversal cuts, with the aid of a steel razor blade, and dissociation of the epidermis from the leaf fragments with the aid of Franklin's solution (Kraus and Arduin 1997). Subsequently, the cuts and epidermis were depigmented with 2% sodium hypochlorite, followed by washing with distilled water and staining with Toluidine Blue. (O' Brien and McCully 1981), Astra Blue 0.12% + Basic Fuchsin 1% (Johansen 1940). The control was the cuts and non-depigmented epidermis. Finally, the cuts were positioned between the slide and cover slip with water, and visualized under a microscope (Zeiss©, Axio Lab.A1), and the images were captured with a coupled trilocular camera (Zeiss©, AxioCam ERc5s).

For the description of the anatomy of the leaf vein, the leaves were depigmented with sodium hypochlorite at a 2.5% concentration, after which they were immersed in Sanfranin 1% for 2 hours. Subsequently, they were washed with distilled water and placed under a Zeiss© magnifying glass for description.

2.5 *Experimental design and statistical analysis*

The experimental design used for the experiments of percentage of protrusion and germination, MGT, CVG and MGV was completely randomized (CRD) with three genotypes (Wild = W; 606 and Santa Fé = SF), with eight replications of 25 seeds for each studied genotype. The imbibition curve was conducted in a CRD and used 15 seeds of each genotype, each seed counting as one repetition. The absolute and relative water content was conducted in CRD, with eight replications of two seeds each, for each genotype. For the biometric analyses of the seeds, a random of 100 seeds per genotype was selected and, for the mass of these seeds, the sampling was of 15 seeds without the tegument per genotype; all the variables were divided into eight equal repetitions of seeds, except for fruits, for which each unit counted as one repetition.

For the leaf anatomy of the seedlings, five leaves were randomly selected from the middle-third region of three seedlings of each genotype; from the median region of each leaf, the midrib, mesophyll, and leaf margin were sectioned. From each cut, three slides were assembled with two anatomical cuts on each. A sampling of the leaf anatomy of the matrices occurred by randomly selecting three matrices of each genotype, from which three branches of each were removed and, from these branches, three leaves were selected for the anatomical cuts and eight leaves to calculate the leaf area. For the description of the midrib, two leaves of the seedlings and three leaves of the matrices of each genotype were used.

The results related to protrusion, germination, germination speed, and biometric analyses were submitted to the Shapiro-Wilk and Levene tests to verify compliance with the assumptions of normality and homogeneity of variances, respectively. Subsequently, a two-way analysis of variance (ANOVA) was performed with the averages of each repetition of the respective analyses to observe the rejection or not of the evaluated hypotheses and, finally, the Tukey test was performed to compare the significant differences between the means.

For the imbibition curve, the homogeneity was verified using the Shapiro-Wilk test; however, the data variance was measured using the sphericity test, using the Mauchly test, and using the Greenhouse-Geisser correction, since the value of the data variance was significant ($p \leq 0.05$). The hypothesis test of this analysis was performed using the ANOVA of repeated means, and the Holm test was used to compare the means. The reduced sampling was due to limited seed availability at the time of collection, and it was necessary to reduce the sample for this analysis for other analyses that required more seeds, such as germination. However, it was above what was necessary to carry out the statistical analysis.

To determine which main variables are associated with each genotype, principal component analysis (PCA) was used, using a correlation matrix to identify clusters or trends in the set of analyzed variables. This analysis used all the quantitative variables for the seeds evaluated in this study.

For the tests related to data normality and heterogeneity, ANOVA and repeated measures ANOVA, Tukey, and Holm were performed using the statistical software JASP© v. 0.17.1 (JASP Team 2023), and the PCA was performed in Past© v.3.16 (Hammer 2021).

7. Results

3.1 *Morphophysiological characterization*

3.1.1 Biometry of the seeds

The three variables (length, width, thickness) showed statistical differences between the three evaluated genotypes (Table 2). The seeds of genotype 606 stood out statistically in relation to Santa Fé and wild seed, for the variables of length, width and thickness and seed mass (p<0.001 for length, width and thickness; p<0.001 for mass of native seed, and p=0.002 for mass of seed of Santa Fé), as well as, the genotype Santa Fé differed statistically from the wild seeds, where all the biometric variables of the genotype were superior, when compared to the wild seeds mass (p<0.001 for length, width and thickness; and p=0.002 for mass of seed).

Table 2 Biometry of seeds of *Bertholletia excelsa.*

Genotype	Length (cm)[1]	Width (cm)[1]	Thickness (cm)[1]	Mass of seed (g)[2]
Wild	35±0.6c	22±0.4c	18±0.3c	3,0±0,3c
606	44±0.9a***	28±0.6a***	22±0.7a***	5.4±0.6a*
Santa Fé	41±0.9b	26±0.6b	19±0.4b	4.3±0.3b

[1] Average based on a sample of 100 seeds
[2] Average based on a sample of 15 seeds
*** p<0.001, * p=0.022

3.1.1 Imbibition curve

The three genotypes showed differences when comparing the averages over the imbibition time. The imbibition of the genotype 606 was higher when compared to the Santa Fé and wild (p=0.002 for genotype Santa Fé, and p<0.001 for wild seed), considering the absolute mass of water in the seed over the imbibition time; as well as, the genotype Santa Fé differed statistically from the imbibition of wild seeds, imbibing more water over the evaluated time (p<0.001).

The imbibition curve followed a pattern for genotypes 606 and Santa Fé, however, for wild seeds, the behavior of imbibition over time was different (Figure 2).

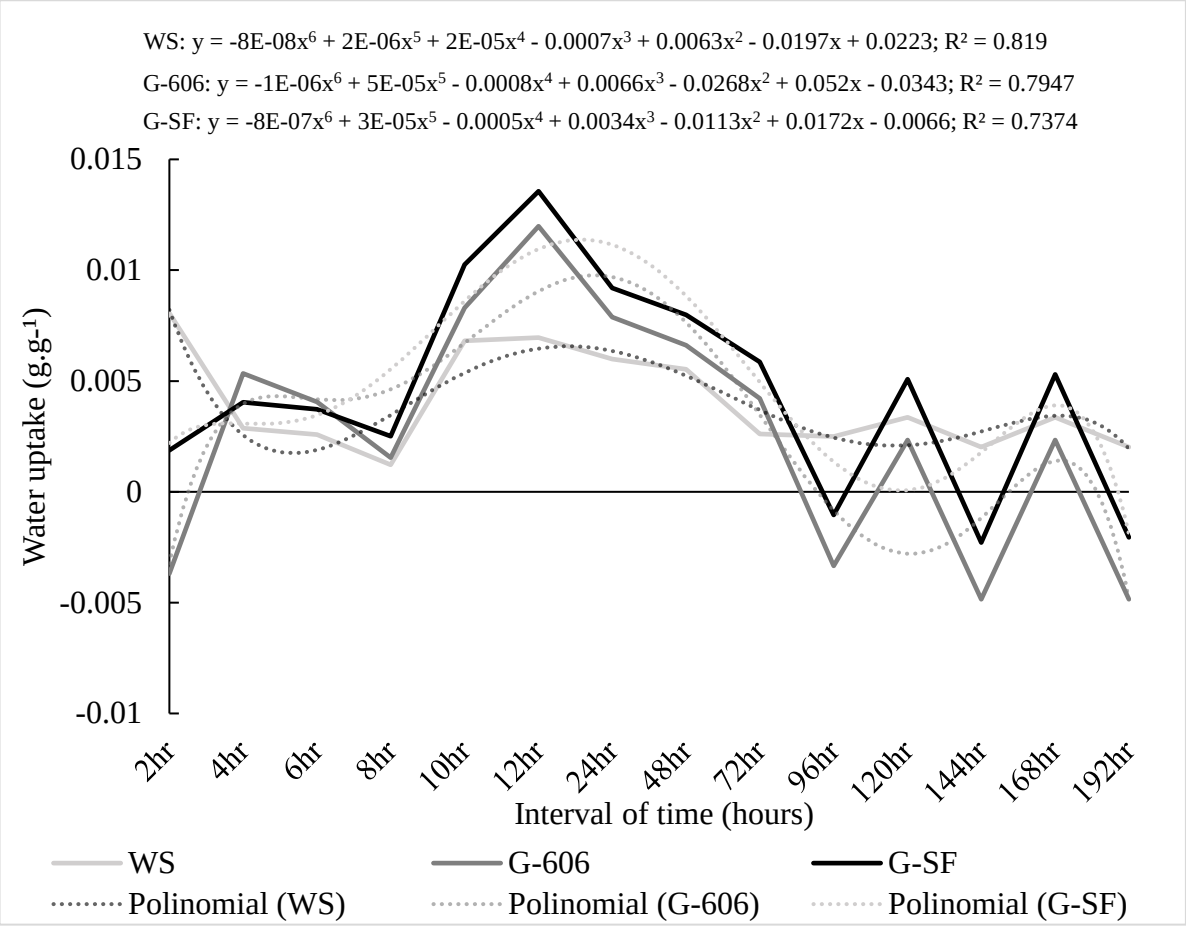

Fig. 2 Curve of imbibition of different *Bertholletia excelsa*'s genotypes. (n=45).

For the genotypes Santa Fé and 606, the comparison between the means of each time evaluated were statistically similar (p=0.3 for 0 h and p=0.4 for 2 h to 192 h), however, when the genotypes are compared with the wild seed, significant differences appear. In the first 72 hours, the genotype Santa Fé has imbibition similar to the wild seed (p>0.06), however, after 96 hours, they begin to differ statistically (p<0.05), while genotype 606 differs statistically from the wild since the first count (p<0.01).

The trend of the curve was similar for both cultivated genotypes. The water absorption values increased from 10 h onwards for genotype Santa Fé, and from 24 h for genotype 606 and wild to 24 h (Figure 3). All genotypes ceased water absorption after 96 h.

Despite the statistical difference between the Santa Fé and genotype 606 during imbibition, when considering seed mass, both have similarities in terms of water absorption over time, taking into account the absolute and relative water mass (p=0.5) (Figure 3b and 3c). On the other hand, both genotypes absorb more water when compared to the wild seed (p<0.001) (Figure 3b and 3c).

Both genotypes and the wild seed showed a positive correlation between the initial mass of the seed and water absorbed by it (Santa Fe: R^2=35.52%, p=0.019; 606: R^2=54.02%, p=0.03; Native: R^2=31.24%, p=0.038); however, when it comes to the relative absorption of water, there

was no correlation (Santa Fe: R^2=0.1%, p=0.907; 606: R^2=0.39%, p=0.830; Native: R^2=27.9%, p=0.053).

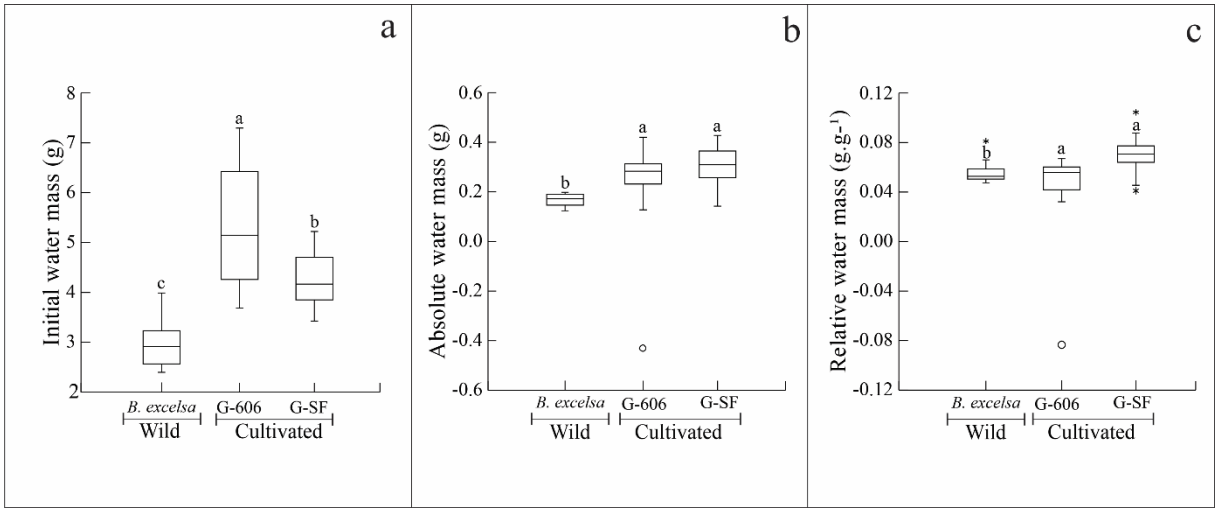

Fig. 3 Absolute and relative mass of uptaken water in seeds of *Bertholletia excelsa*. (a): Initial water mass; (b) Absolute water mass; (c): Relative water mass. (n=15)

3.2 *Germination test*

The protrusion rate was statistically different for the genotypes and native seeds (p<0.001), with 63.3% for 606, 26.5% for Santa Fé, and 5% for native seed (Figure 4a). For the genotype 606, of the seeds that emitted a radicle, only 17.5% grew more than 2 mm in length; for the genotype Santa Fé, this root elongation was 7%, while for the native seed it was only 3% (Figure 4b). The statistical difference also occurred in germination between the genotypes (p=0.028), and between genotype 606 and native seed (p<0.001), however, when comparing the genotype Santa Fé and the native seed, the germination was similar (p=0.26) (Figure 4b). Despite the differences between germination and protrusion between the evaluated genotypes, when taking into account the proportion between the number of seeds that germinated (radicle >2mm), as a function of the number of seeds that emitted the radicle, the values were similar (p>0.5) with 27.5% for genotype 606, 21.8% for genotype Santa Fé, and 45.8% for native seed (Figure 4c).

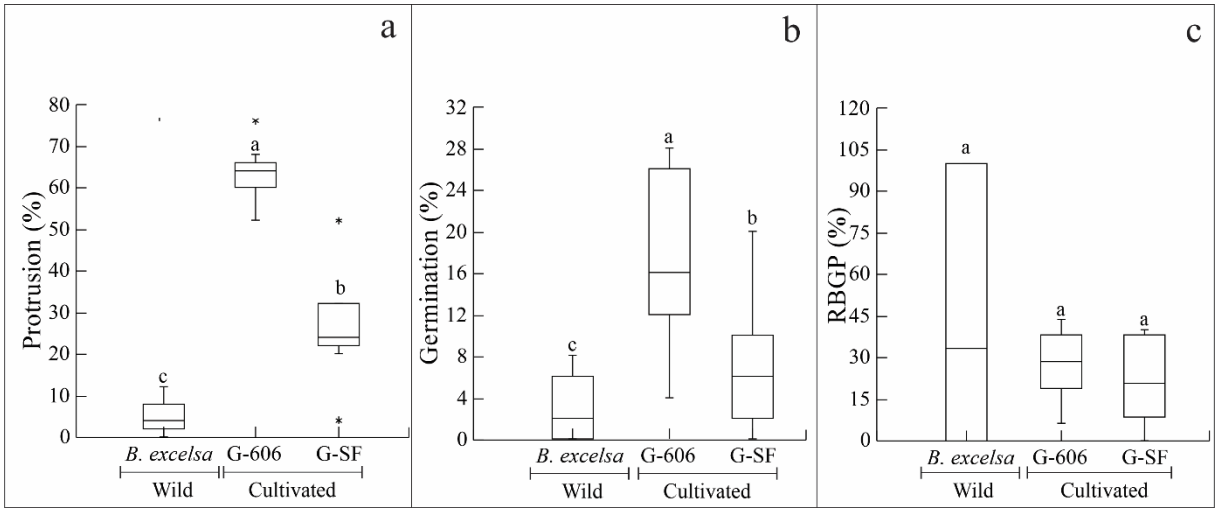

Fig. 4 Germinative parameters of different genotypes of *Bertholletia excelsa*. (a) percentage of protrusion of of the genotypes wild, 606 and Santa Fé (b) percentage of germination of wild, 606 and Santa Fé; (c) RBGP: rate between germination and protrusion. (n=400)

The MGT of the seeds of the wild genotype averaged 58 days, the CVG was 0.008, and the MVG was 0.07 seeds per day. For the genotype 606, these results were 18 days, 0.097 and 0.058 seeds per day respectively, while the genotype Santa Fé averaged 22 days, while the CVG averaged 0.03; and the MGV was 0.02, considering the period of 45 days for cultivated genotypes, and 170 days for the wild genotype (Figure 5a, 5b and 5c).

Despite the statistical differences between the MGT of the genotypes, there was statistical difference for this variable, (p=0.033) for the wild genotype and the 606, although, the wild genotype there was no difference with the genotype Santa Fé (p>0.064), as well as there was no statistical difference between the cultivated genotypes (p>0.9) For the CVG, the wild genotype was similar to the genotype Santa Fé (p=0.09), although, these genotypes were different for the genotype 606 (p<0.001 for the wild genotype and p=0.042 for the genotype Santa Fé) (Figure 5b).

In addition, this comparison was repeated in the MVG variable, as the wild genotype also had similar germination speed with the Santa Fé genotype (*p*=0.145), while genotype 606 germinated its seeds at a higher speed than the other two genotypes (*p*=0.001 for wild genotype; *p*=0.012 for genotype Santa Fé) (Figure 5c).

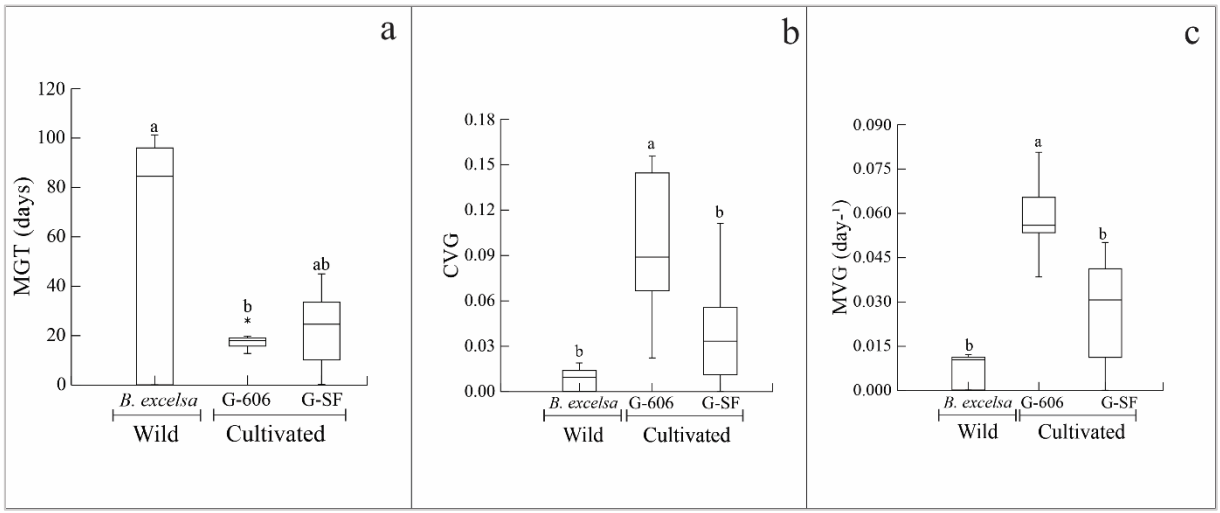

Fig. 5 Velocity of germination parameters of different genotypes of *Bertholletia excelsa*. (a) mean germination time (MGT); (b) coefficient of velocity of germination (CVG); (c) mean velocity of germination (MVG) of the seeds of the genotypes Wild, 606 and Santa Fé and (n=400)

Regarding the main trends observed in this study, it was possible to note that, under the conditions of this study, the gradients that were directly proportional and grouped the three genotypes, as can be seen in figure 6. For the wild genotype, the variable that most represents it is the MGT. For the genotype 606, the following grouped variables were observed: MoW, CVG, %G, MVG, %P and Ls; already inversely proportional, but grouped to this genotype were the length, width of the seed, and the mass of water in the seed. For the genotype Santa Fé were the relative and absolute amount of water, and seed thickness. (Figure 6).

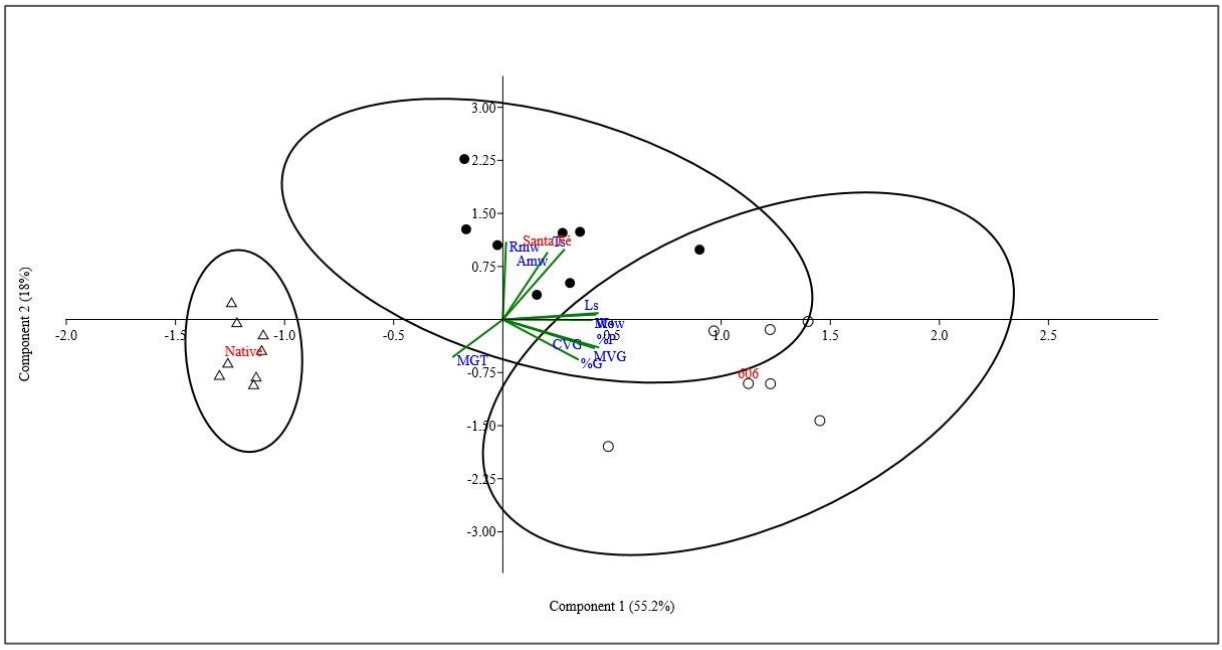

Fig. 6 Principal component analysis (PCA) of the morphofisiology and germination of different *Bertholletia excelsa* seed genotypes, (three variables) wild seed (triangle), 606 (circles) and Santa Fé (black dots). The first two of eleven components (%G=percentage of germination; %P = percentage of protrusion; CVG = coefficient of velocity of germination; MVG = mean of velocity of germination; Mow = mass of water; Rmw = relative mass of water; Amw = absolute mass of water; Ls = length of seed; Ws = width of seed; Ts = thickness of seed) in total are shown, in which PC I explained 55.2% of the total variation in the dataset and PC II explained 18%

3.3 *Morphology of the germination process*

Brazil nut germination can be classified as hypogeal and cryptocodyledonary. The beginning of germination and root protrusion occurred from 15 days after sowing (DAS) for the wild genotype; 16 to 20 days for the genotype 606; and 23 to 33 (DAS) for the genotype Santa Fé, and was marked by the appearance of a greenish color in the seeds, morphologically indicating that it was getting ready to emit the radicle (Figure 7, Af; Bf and Ci). Throughout the cultivated nuts, the formation of callus-like structures was also observed just before radicle emission (Figure 7 Be and Ce).

Subsequently to root protrusion, elongation of the primary root began from 83 DAS for the wild genotype, 28 to 40 for the genotype 606, and 23 to 39 DAS for the genotype Santa Fé, which presented a whitish color and a cylindrical shape with an acuminate apex (Figure 7 Af, Bg and Ch). Following this, expansion of the apex of the root was observed with the establishment of a bulge similar to a cap, and the characterization of a basal portion close to the embryo, with a brown color (Figure Ah, Bi and Ck).

Close to the basal portion of the main root, it is possible to see the protrusion and elongation of secondary roots, also cylindrical, with an acuminate apex and whitish color, which follow the direction of the main root at about 97 DAS for the wild genotype, 69 to 83 for the genotype 606, and 62 to 76 DAS for the genotype Santa Fé, (Figure 7 Ai, Bj and Cl). This suggests the existence of an axial root system, in which the secondary roots originate in a third portion that is called the branching zone.

With the expansion of the root apexes of the roots, a set of hair-like epidermal projections was observed, probably with an absorbent function and which makes up a fourth root portion: the piliferous area (Figure 7 Ak, Bl, Cn). The epicotyl emerged from 135 DAS for the wild genotype, 126 to 170 for the genotype 606, and 105 to 139 DAS for the genotype Santa Fé; and presented a brown color for all genotypes (Figure 7 Am, Bm and Co) For the three genotypes, a morphological region of transition from the root to the epicotyl (traditionally called the collar) during germination (Figure 7 An, Bn and Cp) is not distinguished.

On the stem, growth is monopodial and the apical coloration tends to be greenish at the apex and dark at the base in both genotypes (Figure 7 Aq, Bp and Cr) (Gonçalves and Lorenzi 2011). The phyllotaxis is alternate, giving a spiral aspect to its distribution along the vegetative organ, and it is possible to distinguish the node and internode regions (Figure 8 Ar and Bp) (Gonçalves and Lorenzi 2011) while the leaves originating from the nodes are simple (Figure 8 As and Bq).

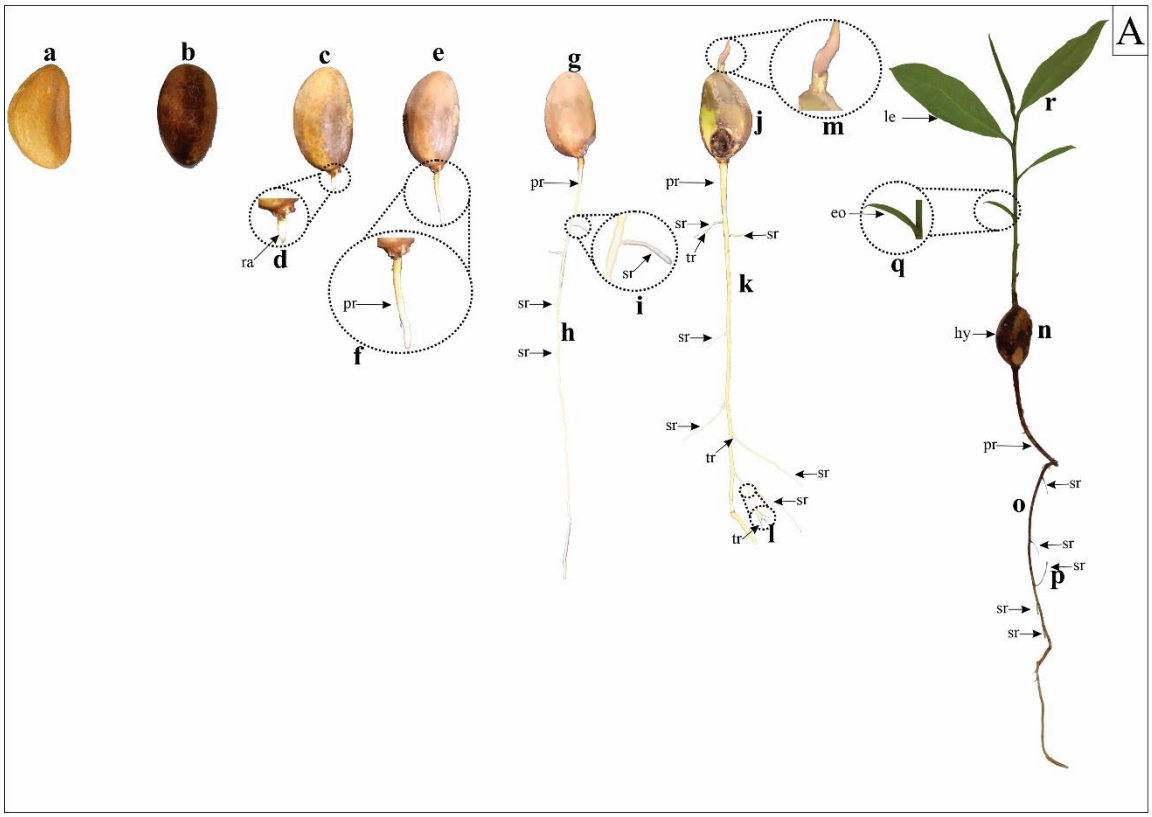


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Fig. 7 Morphology of the germination of different *Bertholletia excelsa* genotypes, (A) Wild genotype; a = quiescent seed; b = imbibed seed; c = seed with radicle protrusion; d = radicle; e = seed with radicle elongation; f = root 1.5mm; g = seed with secondary root; h = primary root; i = secondary root; j = seed with epicotyl emission; k = primary root; l = secondary root; m = epicotyl; n = seed with eophylls; o = primary root; p = secondary root; q = eophylls; r = leaves (B) = Genotype 606; a = quiescent seed; b = imbibed seed; c = seed with radicle protrusion; d = radicle; e = seed callus; f = seed with radicle elongation; g = root 1.5mm; h = seed with secondary root; i = primary root; j = secondary root; k = seed with epicotyl emission; l = primary root; m = epicotyl; n = seed with eophylls; o = primary root; p = (eophylls); q = leaves; (C) Genotype Santa Fé; a = quiescent seed; b = imbibed seed; c = seed with radicle protrusion; d = radicle; e = seed callus; f = callus and greenish coloring; g = seed with radicle elongation; h = root 1.5mm; i = greenish coloring j = seed with secondary root; k = primary root; l = secondary root; m = seed with epicotyl emission; n = primary root; o = epicotyl; p = seed with eophylls; q = primary root; r = eophylls; s = leaves;; ca = callus; eo = eophile; ep = epicotyl; pr = primary root; ra = radicle; sr = secondary root; te = testa; tg = tegmen.

During the germination and early seedling growth, apparent differences were found after the seedlings reached one year of age, mainly in the morphoanatomy of the leaves, in regards to the characteristics of the apexes, margins and secondary veins. The two genotypes have simple and incomplete leaves, composed of the petiole and leaf blade in a dark green color, without the sheath (Figure 8e, 8m and 8u). The blade is lanceolate and glabrous. Venation is eucamptodromous (Gonçalves and Lorenzi 2011).

As to the leaves of mature individuals of the wild genotype, consisting of a slightly winged dark green leaf sheath, a dark green canicular and straight petiole, 1-2 cm long, and an oblong or obovate leaf blade dark green in color, measuring 17-22x 4-7 cm in length, leathery consistency, glabrous surface, with protruding veins towards the lower leaf surface (Figure 8a). The venation pattern is brochidodromous (Figure 8a), the base is convex or acuminate (Figure 8d) and the apex obtuse (Figure 8b). The margin is slightly wavy and crenate (Figure 8c).

As for the leaves of mature individuals of genotype 606, the leaf is complete, consisting of a slightly winged dark green leaf sheath, a dark green canicular petiole, 1-2 cm long, and an oblong or obovate leaf blade. (Figure 98i), dark green in color, measuring 20-28x 5-10 cm in length, leathery consistency, glabrous surface, with protruding veins towards the lower leaf surface (Figure 8k). The venation pattern is brochidodromous (Figure 8k), the base is convex or acuminate (Figure 8l) and the apex rounded (Figure 8j). The margin is slightly wavy and crenate (Figure 8k).

As to the leaves of mature individuals of the genotype Santa Fé, the leaf is complete, consisting of a slightly winged leaf sheath with a light green color (Figure 8q), canicular petiole with a dark green color, 1-2cm long (Figure 8t), and leaf blade oblong or lanceolate in shape (Figure 8q), dark green in color, 24-33x 6-13 cm long, leathery consistency, glabrous surface, with protruding veins towards the lower leaf surface (Figure 8s). The venation pattern is brochidodromous (Figure 8s), the base is convex (Figure 8t) and the apex is concave-cuspid (Figure 8r). The margin is slightly wavy and crenate (Figure 8s).

As can be seen in Figure 8e and 8m, the green coloration is dark for the wild and 606 genotype, while for the Santa Fé, is lighter (Figure 8u). The apex of the genotype wild genotype is attenuated (Figure 8f), the genotype 606 has an acuminate shape (Figure 8n), and for the Santa Fé has an apex acuminated shape (Figure 8v). The leaf margin of the genotype wild genotype is classified as sinuous (Figure 8g), of the 606 genotype is dentate (Figure 8o), with the cuts are more distant from each other, in addition to being larger, and the margin of the genotype Santa Fé is classified as dentate (Figure 8w), similar to the previous one; however, it presents an acute angle in the cuts, in a straight and asymmetrical way, in addition to these cuts

being closer to each other and smaller (Almeida and Almeida 2018). The wild genotype has an attenuated base (Figure 8h), while the cultivated genotypes have a cuneate base (Figure 8p and 8x) (Souza et al. 2016).

The venation morphology of the leaves of the three genotypes has a single primary vein, median, thin towards the apex and thick towards the base (Figure 8g, 8o and 8w). It does not reach the apical margin of the blade. The secondary veins occur in nine pair for the wild genotype, in 25 pairs in the genotype 606 and 18 pairs in the genotype Santa Fé, alternately (Figure 8c, 8m and 8u). The tertiary veins connect to the main or minor veins. Finally, the quaternary veins connect with the primary, secondary, tertiary, or other quaternary veins. The disposition of the tertiary and quaternary veins in relation to the leaf margin gives a brochidrodromous appearance in wild and wild genotype and in 606, and a eucamptodromous appearance in the genotype Santa Fé. The quintenary veins end in other quintenary, quaternary, or tertiary veins, constituting halos.

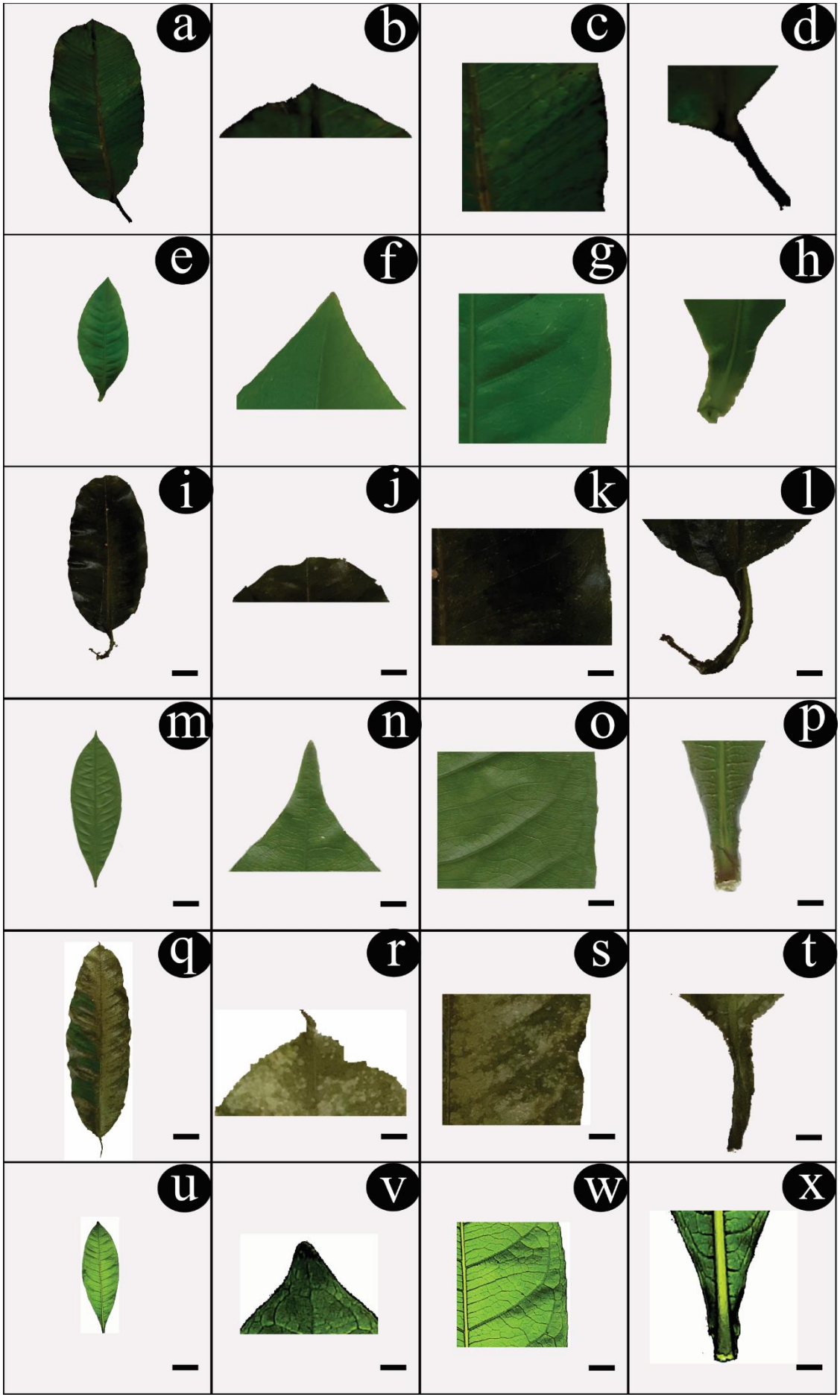
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Fig. 8 Leaf morphology of different *Bertholletia excelsa* genotypes, a-h = wild genotype; i-p = genotype 606; q-x genotype Santa Fé; a) leaf matrix; b) apex of the leaf matrix; c) margin of the leaf matrix; d) base of the leaf matrix; e) seedling leaf; f) seedling leaf apex; g) leaf margin of the seedling; h) leaf base of the seedling; i) leaf matrix; j) apex of the leaf matrix; k) margin of the leaf matrix; l) base of the leaf matrix; m) seedling leaf; n) seedling leaf apex; o) margin of the seedling leaf; p) base of the seedling lea; q) leaf matrix; r) apex of the leaf matrix; s) margin of the leaf matrix; t) base of the leaf matrix; u) seedling leaf; v) seedling leaf apex; w) margin of the seedling leaf; x) base of the seedling leaf.

3.4 *Leaf anatomy*

Regarding the anatomy of the leaves of seedlings of the genotypes, all are characterized, in cross-sectional view, as a uniseriate epidermis, with juxtaposed rectangular cells, on which there is a continuous cuticular surface. On the abaxial surface, they are interrupted by stomatal complexes (Figure 9c, 9i and 9o).

The genotypes have an asymmetric mesophyll, consisting of a layer of palisade chlorenchyma and five to six layers of spongy chlorenchyma, facing the abaxial surface (Figure 9c, 9i and 9o). Under the epidermis of that surface, the substomatal chambers are perceptible (Figure 9c, 9i and 9o). In the midrib region, the mesophyll is without chlorenchyma, consisting of four to eight layers of subepidermal angular collenchyma and fundamental parenchyma (Figure 9c, 9i and 9o).

Regarding the vascular bundles of the wild genotype, they can interrupt the mesophyll, and is of the anfivasal type with a fibrous sheath (Figure 9e and 9f). In the midrib, it is four; two larger medullary and two smaller in eusteles (Figure 9e and 9f), also antivasal and with a fibrous vascular sheath. In the wild genotype, the palisade parenchyma ends at the beginning of the apex of the midrib, while in the cultivated genotypes, this end of the palisade parenchyma cord occurs in the axillary region between the mesophyll and the midrib (Figure 9e and 9f).

As for genotypes 606 and Santa Fé, the vascular bundles of the midrib are the collateral type, however, the genotype 606 has the collar-and-closet type, while de genotype Santa Fé is of the open type (Figure 9k, 9l, 9q and 9r).

The chlorenchyma of the leaf margin of all genotypes is composed of five to seven layers, with palisade and spongy cells not being recognized. Next to it, there is the last vascular bundle of the mesophyll. The angulation of the leaf edge of the wild genotype is slightly angled towards the abaxial side, while the genotype 606 has a sharp angulation towards the adaxial side. The Santa Fé genotype also has the leaf margin facing the abaxial side, however, its angulation is higher than that of the wild genotype

The three genotypes of the species present epidermal cells with thin walls, reacting smoothly to the presence of the toluidine blue dye, evidenced by the slightly bluish coloration. Toluidine blue can still reflect the lilac color under light microscopy when under conditions in which lignin is present in the plant cell (Silva et al. 2014). However, when we observe the cross-sections, the epidermis of the genotype Santa Fé shows a lilac coloration on both sides, subtly indicating a thickening of lignin on the internal periclinal and anticlinal walls, in conspicuous “u”-shaped growth (Figure 9c, 9i and 9o).

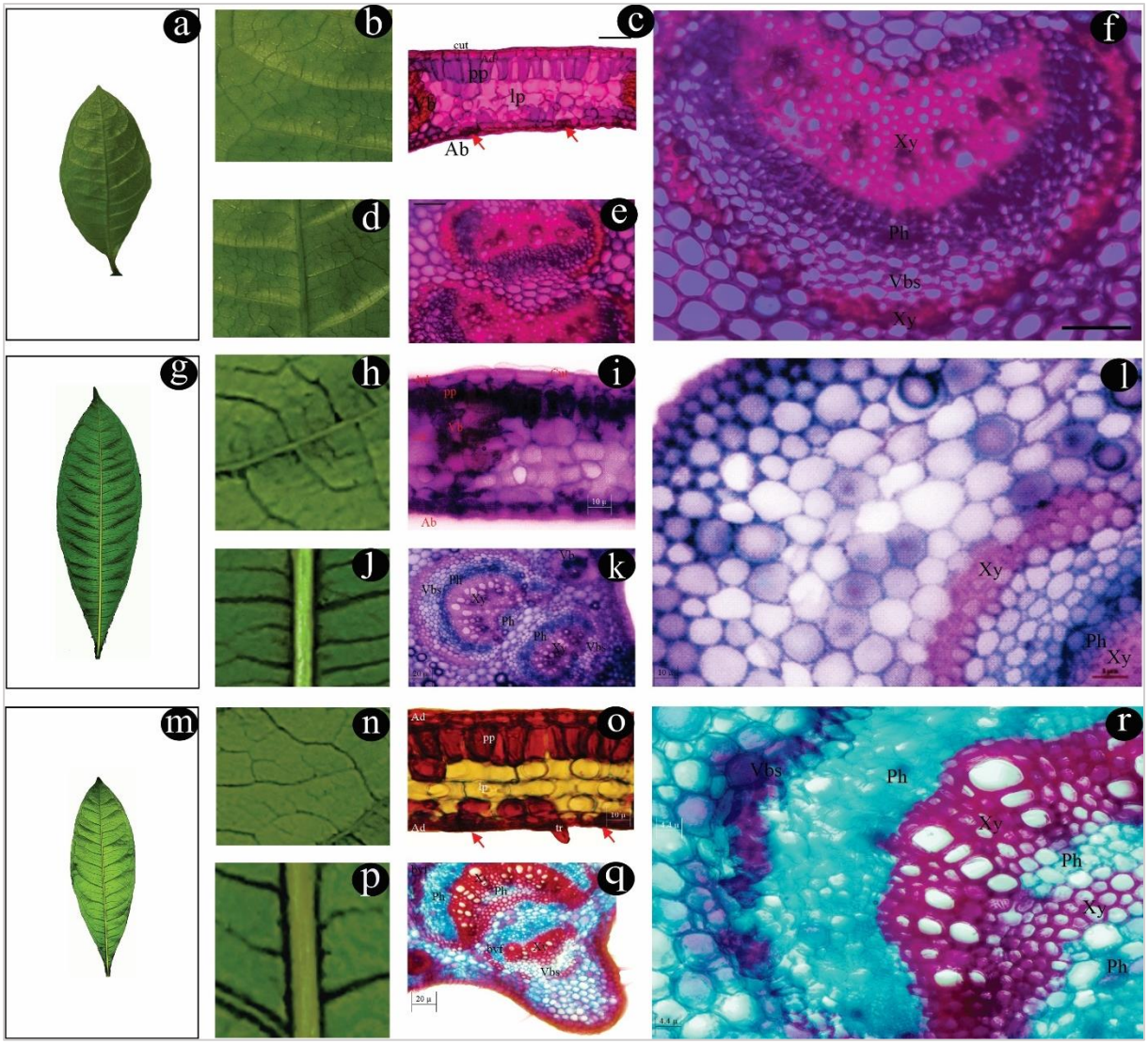

Fig. 9 Seedling leaf anatomy of *Bertholletia excelsa* genotypes, in cross view (c, e, f, i, k, l, o, q and r) a-f = wild genotype; g-l = genotype 606; m-r – genotype Santa Fé. a) leaf; b) leaf mesophyll; c) anatomy of mesophyl; d) leaf midrib; e) vascular bundle of the main vein; f) vascular bundle of the main vein, showing the type of vascular bundle g) leaf; h) leaf mesophyll; i) anatomy of mesophyl; j) leaf midrib; k) vascular bundle of the main vein; l) vascular bundle of the main vein, showing the type of vascular bundle; m) leaf; n) leaf mesophyll; o) anatomy of mesophyl; p) leaf midrib; q) vascular bundle of the main vein; r) vascular bundle of the main vein, showing the type of vascular bundle; Vbs = fibrous vascular sheath; red arrow = stomatal guard cells; lp = lacunous parenchyma; pp = palicadic chlorenchyma; Ad = adaxial epidermis; Ab = abaxial epidermis; Ph = phloem; tr = trichome; xil = xylem.

The stomatal complexes are of the paracytic type and are randomly distributed in the two genotypes, occurring only on the abaxial epidermal surface; therefore, it is a hypostomatic leaf.

8. Discussion

4.1 *Morphophysiological characterization*

4.1.1 Seeds biometry

The results of the biometry of the seeds of the wild and cultivated genotypes 606 and SF reinforce the differences between the studied variables, mainly in the size of the seeds of these genotypes, as well as the mass of the seeds. These measurements can be used as a morphological marker of distinction between the studied genotypes.

When comparing these variables with native matrices, in seeds studied in the state of Mato Grosso, in the central-west region of Brazil, Botelho et al (2019) carried out the biometry of Brazil nut seeds from native forests, found in 4 municipalities, where their averages of length, width, and thicknesses were 40.09, 26.31 and 17.43, respectively. The values express differences between the evaluated variables, with improved genotypes and native Brazil nut matrices.

The use of biometric variables to differentiate genotypes has already been studied. The study by Venial et al. (2017), using seeds of *Theobroma cacao* L., observed differences between the length, width, thickness, and mass of the seeds between the studied genotypes. In the study by Klymenko and Ilyinska (2020), the authors compared the biometry of fruits and leaves of different genotypes of *Cornus officinalis*, and found differences between these parameters, concluding that these differences are the result of climatic variations in the origin of each of the studied genotypes.

4.1.2 Imbibition curve

The trendline of the imbibition curve shows that the apex of the curve is between 12 and 24 hours, for wild genotype, 24 and 48 hours for cultivated genotype. The apex indicates that the maximum point of water absorption occurred at that moment and, after 48 h, the water absorption begins to decline, indicating the stabilization of imbibition. The description of these data shows that phase I of imbibition, in this case, occurs until 48 h, and from that moment on, phase II begins, which ends at 192 h.

This imbibition behavior may indicate that, for the wild genotype, phase I of imbibition occurs within the first 24 hours, and for the cultivated genotypes, this phase occurs within the first 48 h. The phase I is characterized by the rapid absorption of water by the seed in order to restart its metabolic activity. Phase II, which is when the degradation of seed reserves occurs, ends with the protrusion of the radicle (Rajjou et al. 2012; Taiz et al. 2017; El-Maarouf-Bouteau 2022).

It is possible to infer that, in this case, water content, the seed mass positively influences its water absorption; however, the relative water content is not influenced by the initial seed mass. In a study by Zuchi et al. (2012), differences were observed between the imbibition time of different genotypes of *Ricinus communis* L., and these differences were associated with the content of the seeds' reserves, as well as their size. On the other hand, Venial et al. (2017) found no differences between the imbibition of the researched genotypes, even considering the differences between the masses.

In the case of the genotypes evaluated in this study, the seeds of the wild genotype absorbed less water than the cultivated genotypes, both for absolute absorption and for relative water absorption.

4.2 *Germination test*

The proportion between protrusion and germination takes into account the number of seeds that germinated in relation to those that emitted the radicle (Figure 5c). Of the seeds of the wild

genotype that emitted the radicle, 45.8% elongated it, for the genotype 606, this proportion was 27.6% and for genotype Santa Fé 21.8%.

This reflects the statistical similarity between the proportion of root protrusion and germination for the evaluated genotypes. In view of this, it is possible to infer that, despite the difference between the genotypes, and taking into account the rate of protrusion and germination, all are similar when taking into account the set of the two variables. On the other hand, it is important to point out that when the percentage of germination is considered, genotype 606 is superior, when compared with the wild genotype and genotype Santa Fé, under the present conditions of this study.

Of the variables related to germination speed, the one that best reflects the quality of seed germination is the CVG. It is a dimensionless index that expresses the speed of germination and, because it is a variable that is directly proportional to the number of seeds that germinated and inversely proportional to the time that these seeds took to germinate, it is an important indicator of the quality of these seeds (Jones and Sanders 1987; Talská et al. 2020). In this context, the genotype 606 has better germination when compared to the other two genotypes.

The PCA results showed different morphophysiological and germinative groupings between the genotypes, since the wild genotype is more positively associated with the MGT variable, while the 606 is more associated with other variables related to germination (%P, %G, CVG and MVG); the genotype SF is more associated with variables related to morphophysiology (Ts, Ls, Amw and Arw). All cultivated genotypes were grouped close to each other, while native seeds were isolated, indicating, under the conditions of this study, that cultivated and improved genotypes are different from each other.

4.3 *Morphology of the germination process*

The use of leaf venation for systematic and taxonomic characterization of species/groups has demonstrated success in studies with Amazonian clades (Alvarez 2006). However, there are no characterizations of genotypes that apply to the aforementioned anatomical study in order to differentiate infraspecific clades.

In the analysis by Matta (2012), the venation pattern of *Bertholletia excelsa* is brochidrodromous, he does not mention the existence of quinternary veins and states that the endings of the areolas can be branched or not. For the samples analyzed here, the veins do not reach the leaf margin; however, the secondary veins do not end in collector veins, but rather a curve and follow the margin, approaching the secondary vein above, which characterizes a pattern of eucamptodromous venation.

Regarding the absence of citation of quinternary veins in the aforementioned description, all veins, after the tertiary, were considered quaternary. Therefore, the ones here called quinternary are for her quaternary. In sequence, the branching or not of the endings of the areolas were also noticed here, and are repeated in both genotypes.

This condition is related to forest plants, which are inserted in a climax community and stable ecosystem, rarely altered or unstable (Esau 1972).

4.4 *Leaf anatomy*

In recent decades, studies addressing the constitution of leaf anatomy and variation in stomatal percentage have been applied to the delimitation of phenotypes and infra-specific groups, as in the case of the Canadian forest plant *Betula papyrifera* (Betulaceae family) (Pyakurel and Wang 2013). According to these authors, despite leaf anatomy oscillating with the environment and plant response physiology, some cell profiles are selected over time and can characterize genotypes. Thus, it is understood here that, in *Bertholletia. excelsa*, there is a difference in some organization of cells, which indicates a clear morphological characterization of the genotypes here evaluated.

The vascular bundles found in the genotypes are anfivasal (wild genotype) and collateral (of two types, for the cultivated genotypes), i.e., have a xylem unit next to another phloem unit, surrounded by a fibrous vascular sheath. The vascular sheath reacted to the presence of the Basic Fuchsin dye, manifesting a red color. According to Johansen (1940), the reaction of Basic Fuchsin with lignin impregnations is indicated by the red coloration.

Mature vascular bundles may present a sclerenchymatous vascular sheath, when located in parts of the vegetative organ that are susceptible to injuries or injuries, which is a strategy to protect the sap-conducting channel (Esau 1972). Regarding the vascular sheath of the main bundles of the midrib, a more intense reaction to the Basic Fuchsin dye can be seen in the genotype Santa Fé, indicating greater thickening of the cell wall when compared to the sheath of genotype 606 (Figure 9i).

Despite being a controversial subject in plant anatomy for some authors, the application of differences in vascular fiber cell wall thickness is present in the systematic characterization of clades such as *Maxillaria spp*. (Orchidaceae family) (Dettke 2008). These authors performed the differentiation of species of the genus while also considering data regarding the thickening of the walls. For the *B. excelsa* genotypes studied here, we considered the wall thickening of the vascular sheath fibers of the genotype Santa Fé and the thin walls of the genotype 606 characters that also distinguish the anatomy of the seedlings.

Our findings suggest an anatomical differentiation between the leaves of the wild genotype, when compared with the cultivated genotypes, mainly regarding the type of vascular bundle in the midrib, and the place where the palisade parenchyma ends and the collenchyma cells begin at the apex of the vein central. In addition, differences were found between the leaf edge angulation of the three genotypes, suggesting an anatomical marker of differentiation between them.

9. Conclusions

The *Bertholletia excelsa* genotypes (Wild, 606 and Santa Fé) showed morphophysiological differences in seeds, with emphasis on the seed biometry, imbibition curve, and relative amount of imbibed water, percentage of protrusion and germination. Our findings showed that the imbibition behavior and the coefficient of velocity of germination were important parameters to evaluate the morphophysiological differences in germination between the genotypes, highlighting genotype 606, which stood out in all evaluated parameters.

With regard to leaf morphoanatomy, the three genotypes showed divergent patterns regarding the morphology of the apex, base, and leaf margin, which are structures present in seedlings and matrices; as well as in the thickening of the lignin of the periclinal walls, different cells of midrib, termination of palisade parenchyma and shape of vascular bound of midrib, where, this last showed the best parameter to compare the two genotypes. We posit that the morphophysiological and anatomical parameters may serve as markers for differentiating between *B. excelsa* genotypes.

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Chapter 2: Comparative study of the propagation process and the anatomical structure of the leaf of different genotypes of *Bertollethia excelsa* in two cultivation environments [2]

[2] This manuscript will submit to the In Vitro Cellular & Developmental Biology - Plant journal.

1. Abstract

The Brazil nut tree (*Bertholletia excelsa* Bonpl.) provides important ecosystem and socioeconomic services. However, the species is threatened with extinction due to its unconscious exploitation, deforestation, and uneven germination, leading to genetic narrowing. Therefore, alternative measures that enable its propagation are necessary both for mass production and for genetic conservation. In view of this, an attempt was made to establish a protocol for the micropropagation of zygotic embryos, aiming at the production of seedlings with phytosanitary quality for the mass propagation of chestnut tree seedlings. The source material was collected in Itacoatiara-AM and in Parauapebas-PA. The seeds were disinfested with 70% alcohol for 5 minutes and immersed in 1% Amistar WG® and 5% NaClO for 20 minutes, being inoculated in full MS medium (Murashige and Skoog). The results obtained with the zygotic embryos indicated a 40% survival rate and the complete development of seedlings at 84 days. However, despite the promising results, it is still necessary to improve the asepsis protocols for other types of chestnut explants in order to enable in vitro multiplication.

Keywords: Brazil nut tree, plant breeding, seed technology, micropropagation

2. Introduction

Currently, the focus on conservation and recovery of biodiversity in the Amazon rainforest is growing, mainly by government entities that value sustainability (Mansourian *et al*. 2021; Cerullo *et al*. 2023; Freitas 2023; Moutinho and Azevedo-Ramos 2023). However, when the debate is about techniques involving technologies for the production of seeds and seedlings, as well as mass propagation of native species that would serve this purpose, there are few related studies. This is due to the fact that one of the main objects of conservation and recovery are native forest species, which generally take a long time to develop, in addition to the fact that they are more affected by bad weather when they are studied in controlled environments (Bhatt et al. 2023; Castro *et al*. 2023).

Among the important species of the Amazon region, the Brazil nut tree (*Bertholletia excelsa* Bonpl.) is included (Costa *et al*. 2022; Fortes *et al*. 2023; Souza *et al*. 2023). This is due to the provision of ecosystem and socioeconomic services due to its plasticity and exploitation of almonds and wood (Oliveira *et al*. 2021; Costa *et al*. 2022). These services are associated with the adaptation and development of the chestnut tree in different textures and soil fertility, reinforcing its use for the recovery of degraded areas; and with the extraction of almonds, responsible for generating income for traditional communities and rural producers in the Amazon region (Homma 2014).

However, the chestnut tree is considered a vulnerable species by the World Union for Nature (IUCN 2022) and, in Brazil, it is on the list of threatened species by the Ministry of the Environment. The main cause of this threat is the human exploitation of tropical forests that has affected the diversity of tree species (MMA 2014). This causes the suppression of individuals of the species, and consequently, of the genetic variability, associated with the crossing of related individuals and also with self-fertilization (Wadt *et al*. 2018).

With regard to the propagation of the species, it occurs mainly via seeds. However, chestnut seed germination is slow and uneven, with emergence generally occurring after 6 months and extending for more than 18 months after sowing (Auca *et al*. 2018; Dionisio *et al*. 2019; Souza *et al*. 2023). This characteristic is associated, among other factors, with the recalcitrance of the chestnut seed, physical dormancy due to the woody tegument, and physiological dormancy due to the immaturity of the embryo (Dionisio *et al*. 2019; Souza *et al*. 2023).

In order to provide the production of quality chestnut tree seedlings, reduce the pressure on native chestnut trees, as well as guarantee the conservation of the germplasm, it is necessary to use more refined techniques for the propagation of the chestnut tree. In view of this, tissue culture presents itself as a highly effective technique, as it has potential not only for cloning and mass propagation of plant species, but also for the conservation of germplasm of important and rare species (Pawłowski and Staszak 2016; Sánchez 2022).

In vitro propagation allows rapid multiplication of cultivars from vegetative parts (leaves, stems, axillary and apical buds, among others) or zygotic embryos (seeds) of plants, called explants. These

tissues have the ability to dedifferentiate their cells to an initial meristematic stage, allowing, through the induction of growth regulators (synthesized plant hormones), the differentiation of these cells into more specialized ones, such as roots and stems (Abdalla *et al*. 2022; Morinaka *et al*. 2023).

They are inoculated in a culture medium composed of the necessary nutrients for seedling development (carbon source, macro and micronutrients, plant hormones, solidifying agents, among others) (Abdalla *et al*. 2022). This technique allows the formation of seedlings with desirable productive characteristics, such as high productivity, early fruit production, large-scale propagation and with less propagation material, in a reduced physical space, when compared to conventional seedling production (Debnath 2007; Debnath and Arigundam 2020; Abdalla *et al*. 2022; Morinaka *et al*. 2023). Thus, the objective of this work was to establish seedlings of *Bertholletia excelsa* from zygotic embryos in vitro and to adapt in vitro segment multiplication protocols.

3. Material and methods

Origin of zygotic embryos

Bertholletia excelsa genotype 606 seeds were collected from Empresa Agropecuária Aruanã S.A, located in the municipality of Itacoatiara-AM; while the seeds of the wild genotype were collected in the municipality of Parauapebas, in the state of Pará. Soon after collection, they were packed in plastic bags for transport, and were taken to the Laboratory of Plant Physiology and Biochemistry (LFBV), at the National Institute for Research in the Amazon (INPA)/CAMPUS III, located in the municipality of Manaus -AM.

Germinative parameters

The monitoring of the counting of germinated zygotic embryo occurred every three days. Data for two initial stages of germination were collected: Radicle protrusion (RP), defined when the radicle measured 3 mm in length; and radicle elongation (RE), when the radicle measured from more than 5 mm. For each treatment, the germination percentage (G%), mean germination time (MGT) according to Labouriau and Valadares (1976), coefficient of the velocity of germination (CVG), mean velocity of germination (MVG) and percentage of root protrusion precocity (PRPP), adapted from the methodology of Maguire (1962) (Table 1).

Table 1. Equations of germinative parameters

Parameters	Symbol	Equation	Descriptions	Unit
Germination Percentage	%G	%G = (N/A). 100	N = number of seeds germinated; A= number of seeds sown	percentage
Mean Germination Time	MGT	MGT = (Σsgit) / Σsg	t = average incubation time; sg = number of seeds germinated per day; it = incubation time (days)	day

Establishment of seedlings from zygotic embryos

The seed coat was removed with the aid of a knife. Subsequently, they underwent asepsis with 70% alcohol for 5 minutes, followed by immersion in 1% Amistar WG® and 5% sodium hypochlorite for 20 minutes in a flow chamber. Then, they were inoculated in MS Pleno medium, and in the period of 160 days, the development stages of the plant were evaluated until its complete formation at the end of the period (Fig. 1).

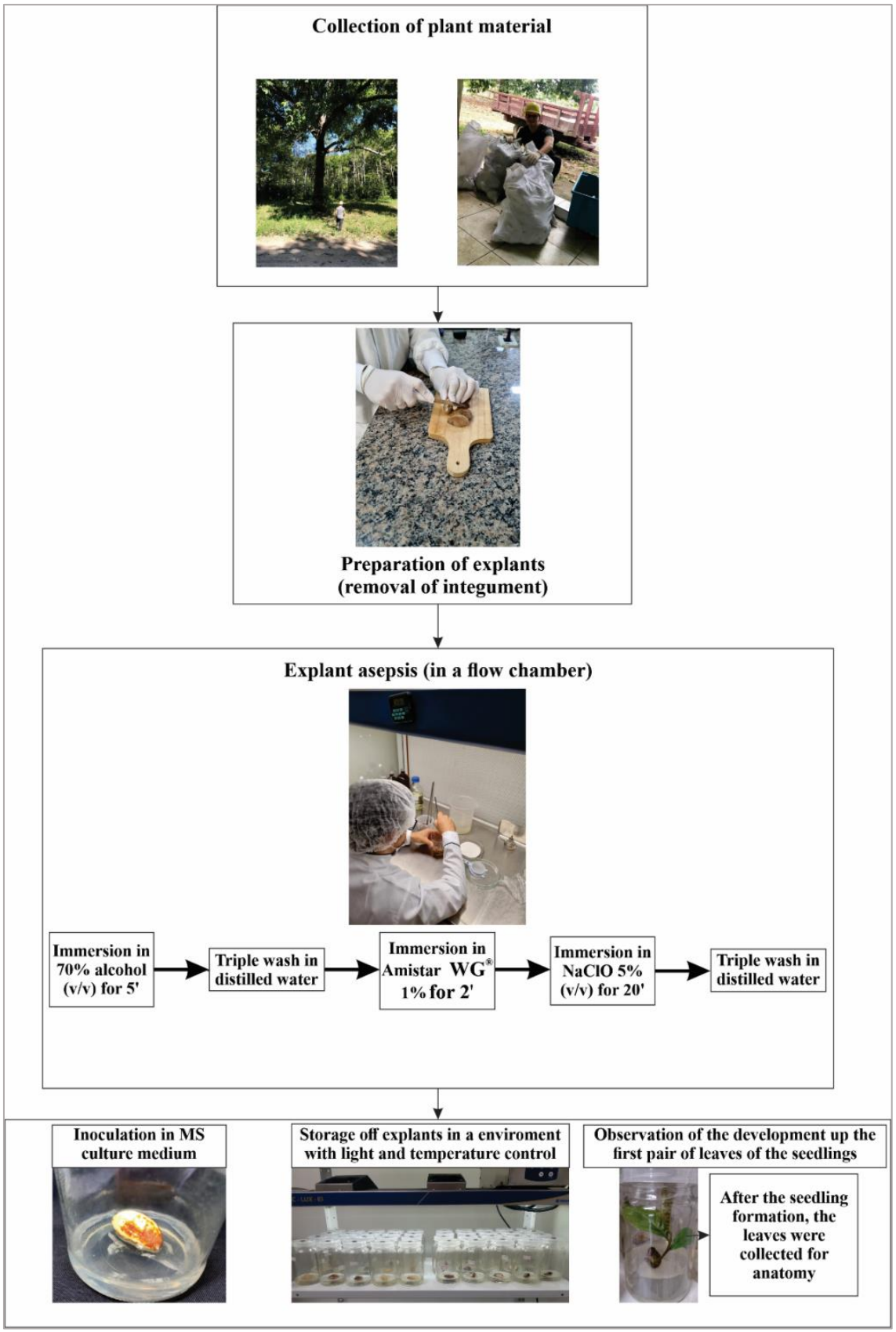

Fig 1. Asepsis protocol and in vitro culture of zygotic embryos of different genotypes of *Bertholletia excelsa*

Germination morphology

The morphological characterization of zygotic embryo and germination was carried out using samples of five units from each stage of germination and seedling formation. Observations were performed with the aid of a Zeiss© table magnifying glass and a stereomicroscope.

For the description, the following variables were observed: emergence and elongation of roots, emergence and elongation of the epicotyl, appearance of eophylls, leaf primordia, and expansion of the first pair of leaves.

Leaf anatomy of in vitro culture seedlings

The leaves were detached from the stem during cultivation. This was followed by freehand transversal cuts, with the aid of a steel razor blade. Subsequently, the cuts and epidermis were depigmented with 2% sodium hypochlorite, followed by washing with distilled water and staining with Toluidine Blue (O' Brien and McCully 1981) + Basic Fuchsin 1% (Johansen 1940). The control was the cuts and non-depigmented epidermis. Finally, the cuts were positioned between the slide and cover slip with water, and visualized under a microscope (Zeiss©, Axio Lab.A1), and the images were captured with a coupled trilocular camera (Zeiss©, AxioCam ERc5s).

Experimental design and statistical analysis

The experimental design used for the experiments of percentage of protrusion and germination, MGT, CVG and MGV was completely randomized (CRD) with two genotypes (Wild and 606), with 20 replications of one zygotic embryo per bottle for each studied genotype.

For the leaf anatomy of the seedlings, five leaves were randomly selected from the middle-third region of three seedlings of each genotype; from the base region of each leaf, the midrib, mesophyll, and leaf margin were sectioned. From each cut, three slides were assembled with two anatomical cuts on each.

The results related to protrusion, germination and germination speed were submitted to the Shapiro-Wilk and Levene tests to verify compliance with the assumptions of normality and homogeneity of variances, respectively. Subsequently, a two-way analysis of variance (ANOVA) was performed with the averages of each repetition of the respective analyses to observe the rejection or not of the evaluated hypotheses and, finally, the Tukey test was performed to compare the significant differences between the means.

For the tests related to data normality and heterogeneity, ANOVA and repeated measures ANOVA, Tukey, and Holm were performed using the statistical software JASP© v. 0.17.1 (JASP Team 2023), and the PCA was performed in Past© v.3.16 (Hammer 2021).

4. Results and discussion

Cultivation of zygotic embryos

After 26 days of inoculation, the first seedling structures began to appear for genotype 606, while for the wild-type genotype, root protrusion started at 40 days after inoculation of the zygotic embryo, as can be seen in Table 2.

Table 2. Time of growth and development of structures in the different germination stages of *Bertholletia excelsa* in vivo and in vitro culture

Germination phase	Days after inoculation/sowing			
	Wild genotype		Genotype 606	
	In vivo	In vitro	In vivo	In vitro
Root protrusion	15	42	18	26
Epicotyl emergence	135	48	148	51
Epicotyl elongation	142	73	163	56
Leaf promontories	156	81	239	60
Expansion of the first pair of leaves	178	104	252	67
Expansion of the second pair of leaves	-	157	466	74

The germination rate for genotype 606 was 25%, where 3 zygotic embryos emitted roots and 2 formed complete seedlings, considering the evaluated time; while for the wild type, the germination rate was 35%. These data are promising, when compared to chestnut tree germination in a in vivo environment, as observed in chapter 1, the germination rate was 17.5% for genotype 606, and 3% for wild type. Some studies also show the low germinability of Brazil nut trees in an external environment, where the highest germination rate found by Dionisio *et al*. (2019a) was 10%, considering a time of 50 days. In case the seed is sown soon after collection, as was the case in this study, the germination rate was 25%, in a study conducted by Silva *et al* (2009), considering an evaluation time of 250 days.

Despite this, the time of emission of the radicle in the in vitro culture increased, since in the conventional production of seedlings, this time was 16 to 20 days, while the radicle emerged at 26 days in the culture medium. On the other hand, the formation of the complete seedling took place after 74 days in the in vitro culture, while in the in vivo culture, this time took an average of 228 days.

As for the wild genotype, in ex vitro cultivation, the average germination time, which takes root emission into account, was 58 days, while in tissue culture, root emergence occurred at 48 days. The first pair of leaves in conventional environment expanded at 170 days, while in tissue culture, it occurred at 104 days.

In a similar study, with the species *Pulsatilla turczaninovii* Kryl. et Serg, Hanus-Fajerska *et al*. (2021) verified differences between in vitro culture environments when compared to ex situ culture. This study corroborates our findings in the present study. On the other hand, there are cases in which

there are no differences between the two cultivation environments, as in the case of Stevia rebaudiana Bertoni, where Yücesan *et al*. (2017) found that both in vitro cultivation and in the conventional seedling production method, the germination rate was 20%. These results show that this difference varies from species to species, even using the same explants.

Despite the survival of the zygotic embryos, 60% of them were contaminated, of which 40% were by fungi and 20% by bacteria, in the zygotic embryos of genotype 606, while for the wild genotype the contamination was 15% by fungi, and 20% by bacteria. Despite contamination in in vitro culture, asepsis was effective, although it needs adjustments. With regard to contamination present in *B. excelsa* explants, some studies have shown that contamination by fungi and bacteria was partial (Pereira *et al*. 2021) and a study by Carvalho *et al*. (2013) observed 5% contamination, recommending the use of 5% calcium hypochlorite in the asepsis process due to its high efficiency in the disinfestation of immature seeds of *Bertholletia excelsa*.

Morphology of in vitro germination

After 28 days after inoculation (DAI), the zygotic embryo of the wild type showed a green color in the region of the cauline and root poles. Then, the shade of green color was darkening, and it acquired a shiny appearance. The first signs of calluses in the embryos occurred soon afterwards, and from that moment on, the radicle emitted at 42 DAI, with a whitish color (Fig. 1a).

After elongation of the radicle, at 48 DAI, the zygotic embryo emitted the epicotyl (Fig. 1d). After its elongation, there was the formation of leaf primordia, of light green color (Fig. 1e). Then, the beginning of the development of the first signs of the second leaf, arranged alternately in relation to the first, was observed (Fig. 1e). The second leaf expanded before the first leaf, and after its full expansion the first leaf continued its expansion (Fig. 1e).

Concomitant with the growth of the epicotyl and expansion of the first pair of leaves, the embryo completely acquired a light and fark green pigmentation (Fig. 1f).

With regard to genotype 606, shortly after inoculation, the embryo showed a dark green color with dotted brown bands at the longitudinal ends, throughout the explant, before root protrusion (Fig. 1e). Before the emission of the radicle, there was the formation of random whitish calluses along the embryo, and on the root pole, which darkened during root elongation (Fig. 1h).

Initially, the radicle presented a whitish color, shortly after emergence, and later, it was pigmented in green during its elongation. The base region of the root is thick and lignified, while the apex is less thick than the base and milky white in color. (Fig. 1i).

After elongation of the radicle, epicotyl emission occurred. Initially, the leaf primordia and the epicotyl showed a brown color. The epicotyl was thickest in the region close to the embryo, and thinnest

in the region close to the leaf primordia (Fig. 1k). The leaf primordia presented similar coloration to the epicotyl and it was poorly developed.

The second leaf has an alternating disposition to the first, however, its expansion happened first, acquiring a dark green color, with a leathery leaf blade after its total expansion. Then, the first leaf of the stem expanded, with the leaf blade also dark green and leathery (Fig. 1l).

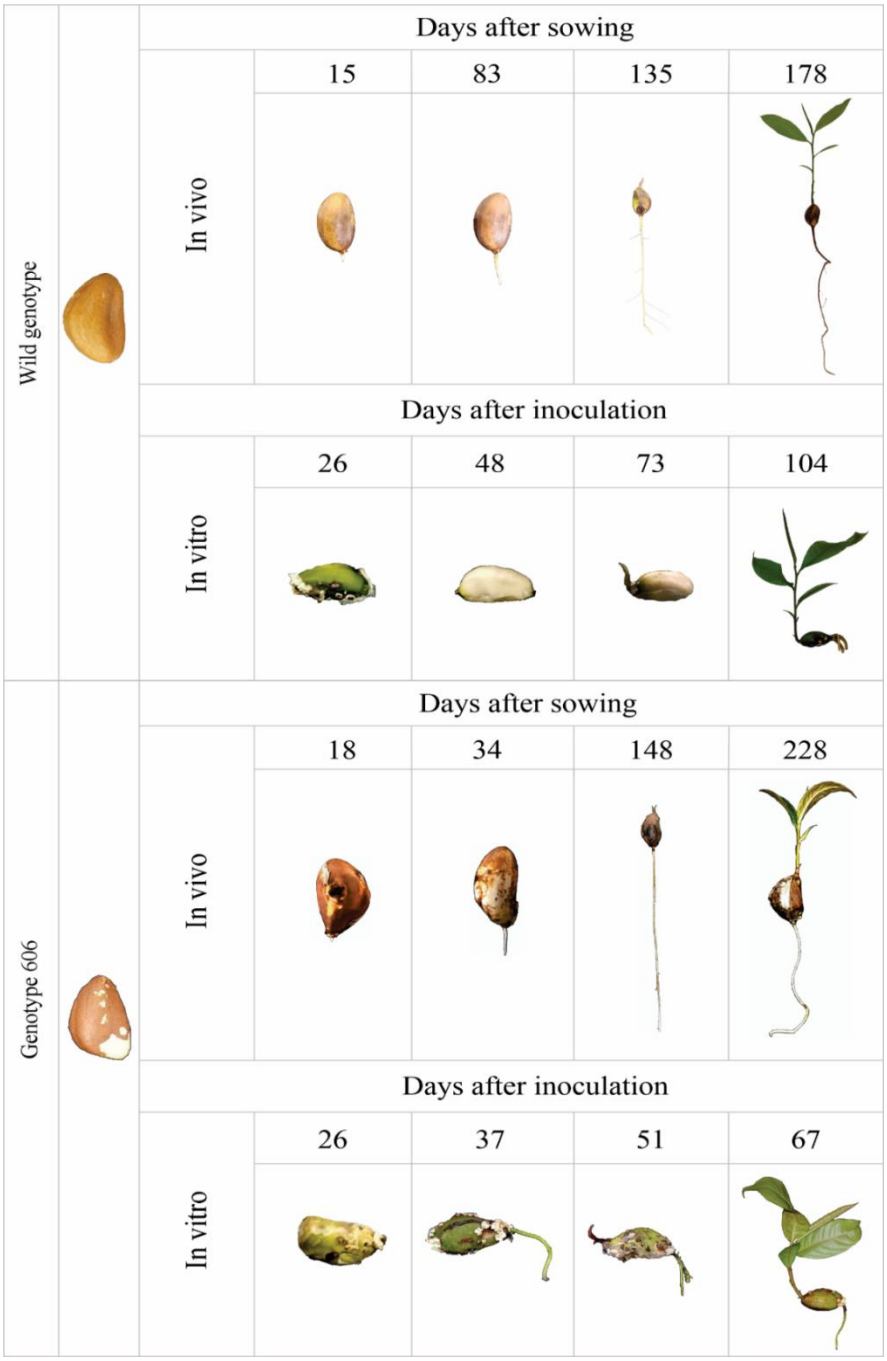

Figure 1. Growth and seedling development of different genotypes of *B. excelsa* in vivo and in vitro

During the development of the embryos in the culture medium, differences were noticed during the germination of the two genotypes. While in the wild genotype calluses occurred only at the poles of the hypocotyl-radicle axis, these structures occurred more frequently in genotype 606, in addition to the entire region of the embryo. Calluses may be associated with a large number of meristematic cells that form the embryo of the species, causing disordered growth and development of these tissues, causing these calluses (Camargo *et al*. 2000; Santos *et al*. 2013) Another aspect that stood out among the genotypes was the color of the embryo, taking into account both color intensity and brightness. These findings corroborate the data found in chapter 1, where it was possible to observe that the green pigmentation of the seed before the radicle emission also occurred for the wild and 606 genotypes, in addition to the presence of calluses along the embryo, during the seeding process. germination. However, in in vitro culture, these characteristics were more evident, since in the culture medium, the entire embryo and the root turned green.

Studies by Carvalho *et al*. (2022), did not observe significant differences in the morphological characteristics between the studied genotypes of *Hevea* spp in vitro. The fact is that these differences can occur according to each species and/or genotype studied and can also be influenced by the environment. This influence of the environment could be observed in plants of *Hyssopus officinalis* f. *cyaneus* Alef (Common hyssop), grown in vitro and ex situ, and there were differences in leaf blade morphology and cuticle thickness in these different environments (Plugatar *et al*. 2023).

The green pigmentation of the zygotic embryo during germination was also observed by Carvalho *et al*. (2023), in wild and cultivated genotypes of the genus *Hevea* sp., propagated in vitro, however, pigmentation of these embryos did not occur in the in vivo culture (Carvalho *et al*. 2022; Carvalho *et al*. 2023).

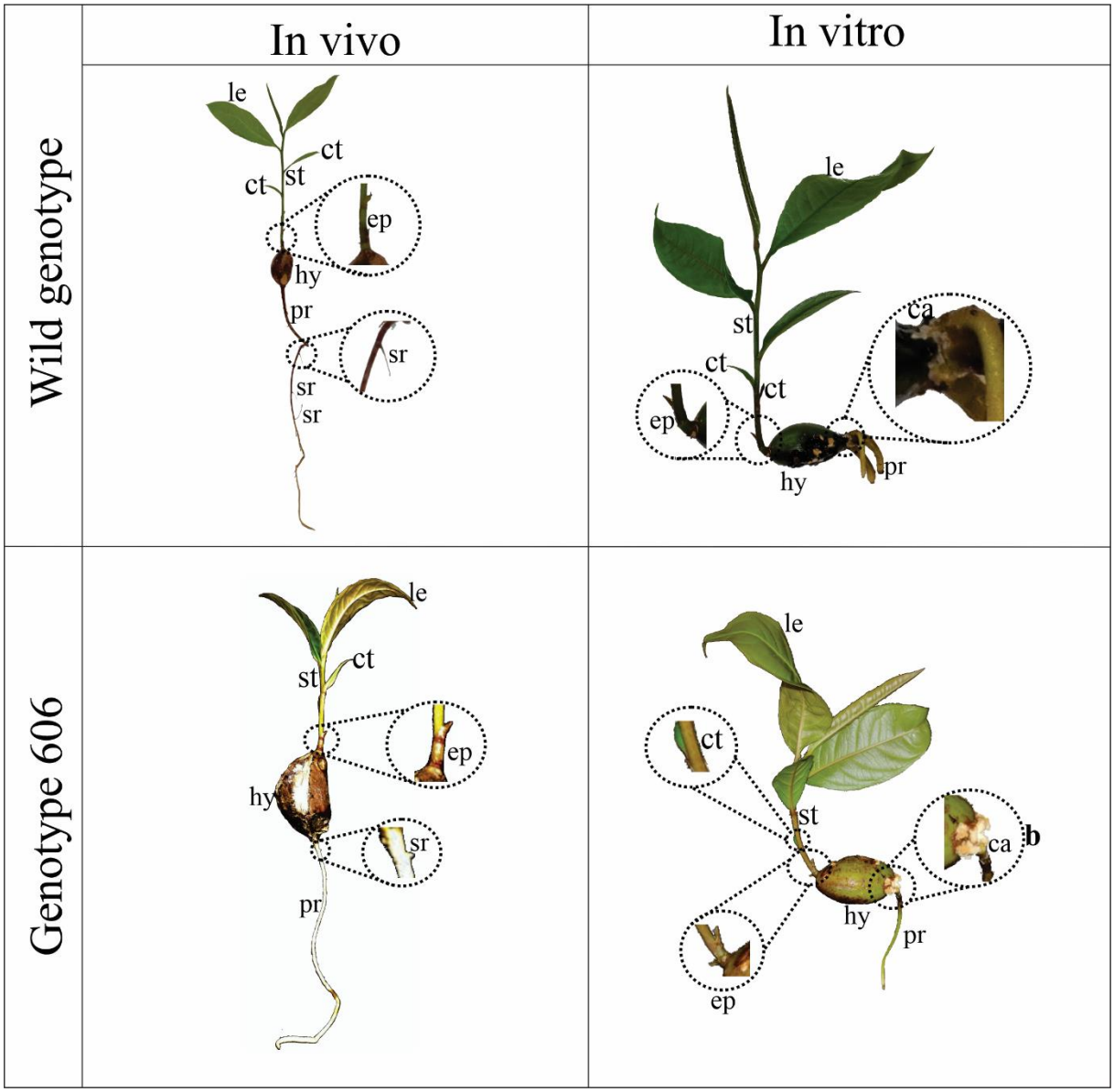

Figure 2. Morphology of seedlings and leaves of different genotypes of *B. excelsa* in vivo and in vitro, hy = hypocotil; pr = primary root; sr = secondary root; ep = epicotyl; st = stem, ct = cataphylls, le = leaf, ca = callus

Leaf anatomy of seedling grown in vitro

On the leaf margin of the seedling formed in vitro, of the wild genotype, it is possible to observe that its curvature is turned downwards (Fig. 3g). The palisade parenchyma starts right after the first vascular bundle (Fig. 3g). In the midrib of this genotype, the vascular bundle close to the adaxial surface has a collateral pattern and an oval shape (Fig 3i). The central region of the vascular bundle is filled with collenchyma cells (Fig. 3i).

In the region close to the apex of the adaxial face of the leaf, in the midrib, the palisade parenchyma originating from the leaf mesophyll ends (Fig. 3j). Just below the epidermis, in the upper region of the collenchyma, there is only one layer of epidermal cells (Fig. 3j)

Leaf epidermal cells of genotype 606, formed in vitro, divide periclinally, and are formed by a single layer of cells, just below the cuticle (Fig 3r). The number of layers of the adaxial and abaxial epidermis is the same. When at the margin, the epidermal cells have a square shape and when they are distributed along the mesophyll, they have a horizontally elongated shape (Fig 3r).

Just below the epidermal cells, the spongy parenchyma occurs, with the palisade parenchyma absent in the region of the leaf margin. The cells of the spongy parenchyma have cellular content, which may be starch (Fig. 3s). In the region of the vascular bundle, there are 5 layers of hypodermic cells, in the abaxial region, and two hypodermic layers in the adaxial region; phloem vessels surround xylem vessels in a “u” shape; the central vascular bundle is adjacent to another vascular bundle on the right side, with smaller cells, with the phloem surrounding the xylem (Fig. 3t and 3u).

The vascular bundles, central and adjacent, are surrounded by the spongy parenchyma, having a concentric pattern and “w” shape. In the region of the abaxial epidermis, there is the formation of trichomes (Fig. 3t).

Along the mesophyll, after the leaf margin and between the secondary venation, the palisade parenchyma occurs in the adaxial region. In the region of the spongy parenchyma, there are schizogens (intracellular spaces) (Fig. 3s).

At the intersection between the epicotyl and the leaf, just below the epidermal cells, there is the presence of lamellar collenchyma in a continuous band, in the peripheral portion of the young petiole (in formation in the seedling). As the cells develop towards the central region of the structure, the collenchyma has less thick cell walls than those found near the primordia, being then classified as annular collenchyma (Fig. 3u).

In the central region of the vascular bundles of the midrib of the leaf of genotype 606, there is a secretory gland, and the xylem just above it, facing the adaxial face of the leaf, has a “w” shape, as well as the phloem cells that secrete it surround (Fig. 3t).

In the region below the epidermis of the midrib, the palisade parenchyma has an isodiametric shape, and is present even in the apex region, where it meets with collenchyma cells. Just below the epidermis at the apex of the midrib, in this genotype there is the presence of a subepidermal layer (Fig. 3r)

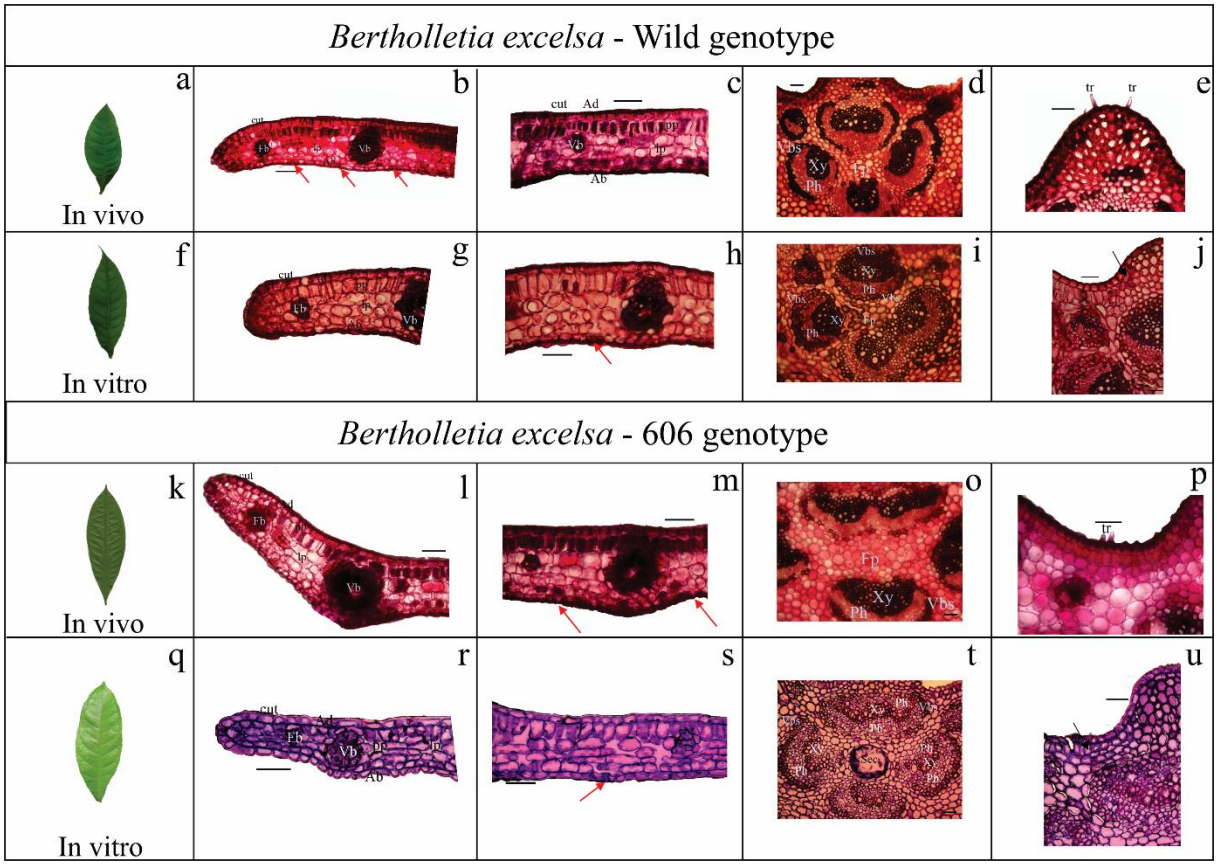

Figure 3. Leaf anatomy of seedlings of different genotypes of *Bertholletia excelsa* formed in vivo and in vitro culture, seedling leaf (a, f, k and q); anatomy of the leaf edge (b, g, l and r) anatomy of leaf mesophyll (c, h, m and s); midrib (d, i, o and t); apex of midrib (e, j, p and u); cut = cuticle, ad = adaxial epidermis, ab = abaxial epidermis, pp = palisade parenchyma, lp = lacunous parenchyma, Fb = fibrous vascular bound, Vb = Vascular bound, Vbs = vascular bundle sheath, Ph, phoem, Xy = Xylem, Fp = fundamental parenchyma, tr = trichomes, Sec = secretory cavity; the red arrows indicate the stomatal chamber, the black arrows indicate the end of the palisade parenchyma originating in the mesophyll

When comparing the two genotypes, it is possible to detect some differences in leaf anatomy (Table 3).

Table 3. Anatomical differences in vitro formed leaves of different genotypes of *Bertholletia excelsa*;

Anatomical character	Wild genotype		Genotype 606	
	In vivo	In vitro	In vivo	In vitro
Starch grain size	Conspicuous	Conspicuous	Conspicuous	Incospicuous
Amount of starch grains in the palisade parenchyma	Numerous	Scarce	Numerous	Scarce
Collenchyma in midrib	Angular	Angular	Ring shape	Ring shape
Termination of palisade parenchyma	Concavity of the adaxial midrib	Concavity of the adaxial midrib	Axilla of the midrib	Axilla of the midrib
Type of vascular bundle of the midrib	Collateral	Collateral	Bilateral	Bilateral
Filling of the medulla of the midrib	Fundamental parenchyma	Fundamental parenchyma	Fundamental parenchyma	Secretory cavity
Leaf edge	Slightly flexed to the abaxial face	Slightly flexed to the abaxial face	Angular facing adaxial face	Straight
Epidermal angulation of the leaf edge	Hemiovallate	Hemiovallate	Acuminate	Acuminate
Leaf edge filling	Palisade and lacunous chlorenchyma	Palisade and lacunous chlorenchyma	Palisade parenchyma	Undifferentiated chlorenchyma

Legend: in yellow = equal between genotypes and in vivo and in vitro cultures; in orange = equal between genotypes and different in vivo and in vitro cultures; in blue = different between genotypes and the same in vivo and in vitro cultures; in purple = different between genotypes and different in vivo and in vitro cultures

It was possible to observe eight anatomical differences in the leaves of the two genotypes in in vitro cultivation, varying between the region of the midrib, mesophyll and leaf margin, as can be seen in Table 3. A similar anatomical comparison between wild and domesticated genotypes was performed by Lei *et al*. (2021), using cotton leaves (*Gossypium* sp.) formed in vitro, and in this work quantitative differences were verified between the cell wall thickness and the size of the mesophyll cells of the two genotypes.

Another similar comparison was evaluated by Leite *et al*. (2023), using different genotypes of heliconias (*Heliconia rauliniana*; *Heliconia bihai* cv. Lobster Claw Two, and *Heliconia. rostrata*), using the technique of micropropagation by grafting, and differences were detected in the anatomy of the leaves between these genotypes, mainly in thickness of the mesophyll, palisade parenchyma, spongy parenchyma, and in the adaxial and abaxial epidermis of these genotypes, under the evaluated study conditions.

In addition to the anatomical difference in vitro cultivation between the genotypes, differences were also observed between the different forms of propagation carried out in this study. In both

genotypes, there was a higher frequency of trichomes in the region of the midrib in the in vivo culture than in the in vitro culture. The difference in the occurrence of trichomes in different forms of propagation was also detected by Mani and Shekhawat (2017), where, in leaves of seedlings of *Passiflora foetida* L., cultivated in vivo, they developed more trichomes than in in vitro cultivation. In addition, a greater presence of palisade chlorenchyma filled with some substance, which could probably be starch, was noted in leaves formed in vivo than those formed in vitro.

For the genotype 606, only the vein close to the adaxial face is classified as bilateral, while the adjacent veins are all collateral; and unlike in vitro culture, the central region between the veins is filled with collenchyma cells; finally, the marginal cells in the abaxial region are organized irregularly in ex vitro culture, while in in vitro culture, this region is regular, forming a semi-sphere. The leaf edge presented an angular curvature towards the adaxial face of the leaf.

Plugatar *et al*. (2023) also found anatomical differences in the leaves of *Hyssopus officinalis* cultivated in vitro and in vivo, where the main differences evidenced were in the thickness of the cuticle, in the adaxial and abaxial epidermal cells, and morphology of the palisade and spongy parenchyma found in the leaf mesophyll.

As it was possible to observe, the anatomical differences that group different genotypes of the same species can be detected in the leaves, in different sections that form it, regardless of the form of propagation, whether vegetative or reproductive, therefore, under the conditions of this study, it was verified that in addition to the possibility of propagating the Brazil nut tree using the in vitro cultivation technique, as a way to enhance the production of seedlings in less time, it is possible to group different genotypes of the species through the anatomy of the leaves, mainly in the midrib region, mesophyll and margin.

5. Conclusion

The in vitro establishment of plants of different genotypes of *Bertholletia excelsa* (wild and 606) proved to be efficient for explants of zygotic embryos, as they presented better performance in terms of development time, low contamination and high germination rates, when compared to the conventional cultivation. The development of the two genotypes showed different behavior, mainly regarding morphology and time of germination.

The two genotypes showed distinct leaf morphoanatomical characteristics, mainly in the midrib and leaf margin.

Despite the positive results obtained, the in vitro establishment of plants from zygotic embryos still requires adjustments for other types of *B. excelsa* explants, in order to obtain an efficient asepsis protocol for in vitro multiplication.

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7. GENERAL CONCLUSION

The study of the germination of different Brazil nut genotypes (*Bertholletia excelsa* Bonpl.) from the morphophysiological and anatomical point of view of seeds, suggests advances regarding the genetic improvement of the species, taking into account the different results of these genotypes in terms of seed biometry, absorption of water, and germination parameters.

When qualitative variables related to leaf morphoanatomy are taken into account, it is also possible to distinguish these different genotypes evaluated here, reinforcing the idea of specific characteristics that belong to each of these genotypes.

In view of this, our findings suggest morphophysiological and morphoanatomical markers that can be used to practically differentiate wild genotypes from cultivated Brazil nut trees. In addition, through our results, it was possible to select genotypes that are more efficient for the production of seedlings, which can be used for different purposes.

Another relevant point in this research is the advancement in techniques that can improve the production of quality seedlings, and more quickly, through in vitro micropropagation of zygotic embryos.

Despite our advances, there are gaps regarding the slow and uneven germination of the species that still require a more in-depth and refined investigation, as well as the investigation of asepsis protocols in the culture of plant tissues for the species, which are more efficient, and in addition, the use of other explants for in vitro micropropagation.